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THE TRANSPORT OF CO_2 IN THE BLOOD OF CERTAIN FRESHWATER FISHES

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The effect of carbon dioxide (and other acids) on the affinity of the hemoglobin in fish blood for oxygen varies greatly from species to species (Redfield, 1933). In some species not only the affinity of the blood for oxygen, but the oxygen capacity too, is greatly diminished by relatively low pressures of CO_2 (Root, 1931). The physiological and ecological implications of great differences in affinity for oxygen and sensitivity to CO_2 have become more apparent as a result of a series of studies on freshwater fish, which are summarized in a recent paper by Black (1940). A few of the findings may be recapitulated briefly as follows. Carbon dioxide decreases the affinity of the blood for oxygen to a greater degree in those fish inhabiting deeper and colder water. The same bloods have also a lower affinity for oxygen at minimal pressures of CO_2. These two characteristics would act to offset the effect of low temperatures, which is to lower the pressure at which oxygen is available to the tissues. Another manner in which sensitivity to CO_2 may be useful to fish inhabiting deep water, is in the regulation of buoyancy at different depths, by facilitating the formation in the swim-bladder of gases rich in oxygen, as suggested by Haldane (1922) and Hall (1924).

The present study was designed with two objects in mind. The first was to investigate the mechanisms by which the great differences in sensitivity to CO_2 are achieved. The second was to determine the probable range of physiological tensions of CO_2 in a series of freshwater fish comprising some of those used in the foregoing studies. This information is difficult to obtain with any degree of accuracy in fish, but even approximate determinations may be of use in evaluating some of the relationships described above.

In pursuit of the first object, a detailed study of the characteristics

of CO_2 transport was made on the blood of two species differing widely in the sensitivity of their blood to CO_2, namely rainbow trout, *Salmo gairdnerii* Richardson, and carp, *Cyprinus carpio* Linnaeus. Trout are among the fish most sensitive to CO_2 and carp, while not the most insensitive, are relatively so. Availability in sufficient numbers to supply enough blood was an important consideration in the choice of these species. Data on physiological CO_2 tensions were obtained on the same species as well as on a few specimens of bullhead, *Ameiurus nebulosus* Le Sueur, and sucker, *Catostomus commersonnii* Lacepède, which happened to be available. The blood of the bullhead is even less sensitive to CO_2 than that of the carp, while those of the sucker and the trout are of about the same order of sensitivity.

Methods

Blood was drawn from the heart into a syringe containing heparin. No fluoride or oxalate was used, since these have been found to cause progressive swelling of the erythrocytes and eventual hemolysis in certain fish bloods (Black and Irving, 1938; Hamdi and Ferguson, 1940). For the construction of dissociation curves it was necessary to pool the blood from several fish. Equilibration was done at 15° C., except in specified cases. The blood was kept on ice throughout the day. Samples were equilibrated one at a time for fifteen minutes in tonometers of the original Barcroft type (1914). A centrifuge tube was attached to the open end of the tonometer by rubber tubing. After equilibration the blood was collected in the centrifuge tube and separated from the gas phase by clamping the rubber tubing. The gas was analyzed for CO_2 and O_2 in a Haldane apparatus. The blood in the centrifuge tube was covered with liquid paraffin and samples were removed without delay for analysis of CO_2 and O_2 by the manometric method of Van Slyke; of chloride by the open Carius method; and of water content by drying at 105° C. Another sample was centrifuged in a capillary tube for twenty minutes at a centrifugal force of about 3000 times gravity for the estimation of packed cell volume. The rest of the blood was also centrifuged under oil for 20 minutes and samples of plasma removed for the analyses listed above. A number of experiments were performed to evaluate the error introduced by metabolism of the blood during the foregoing procedures. Hourly analyses were done on blood kept in sealed syringes at 15° C. and on ice. Trout blood was found to have a somewhat higher metabolic rate than carp blood, and the highest rate found in trout blood at 15° C. was 1 cc. of oxygen consumed and 1 cc. of CO_2 produced per 100 cc. blood per hour. The only estimation which

might have been appreciably affected by even this highest metabolic rate was the estimation of cell volume in which centrifuging was done at 25° C. or thereabouts for 20 minutes. It was possible in the later experiments on trout blood to minimize this source of error by reducing the time of centrifuging to 5 minutes in a high speed hematocrit centrifuge. The results with both long and short times of centrifuging were in substantial agreement.

In trout blood kept on ice, the highest metabolic rate was found to be 0.5 cc. per 100 cc. per hour. This figure may be used to estimate the maximum correction for the figures on O_2 and CO_2 content of the venous blood of trout, because the trout blood was kept on ice for three or four hours in transit from the hatchery to the laboratory.

Some experiments were done to determine the change in CO_2 capacity with time. No changes were found in blood kept on ice for as long as 12 hours. When the blood was kept at 10° C. in a refrigerator for 24 to 48 hours, small changes occurred. It is interesting that the CO_2 capacity increased in blood (both carp and trout) kept fully oxygenated, but decreased in blood which was partly or fully reduced, suggesting something like a Pasteur reaction.

The gas content of mixed venous blood, drawn from the heart into an oiled syringe containing heparin, was determined by analysis without exposure to air. Three portions of the same sample were then equilibrated with different gas mixtures and subsequently analyzed. One portion was equilibrated with about 2 mm. CO_2 in air. This gave the oxygen capacity and one point on the CO_2 dissociation curve. Another portion was equilibrated with 8–12 mm. CO_2 in air and the third portion with 8–12 mm. CO_2 in N_2. These gave the positions of the CO_2 dissociation curves of oxygenated and reduced blood respectively. The equilibrations in these particular experiments were done at the temperature of the water from which the fish were taken. Knowing the oxygen content of the sample of venous blood and the oxygen capacity of the same sample, it was possible to estimate the position of the CO_2 dissociation curve of the venous blood between those of the fully oxygenated and fully reduced bloods. Then knowing the CO_2 content of the venous blood, its CO_2 tension could be read off on the abscissa with an error probably not more than 2 or 3 mm. Hg.

During the withdrawal of the venous blood, the gills were not aerated, but only samples which flowed freely into the syringe at the first stab were used. Since even the trout survived this procedure without apparent harm, the estimated tensions may be regarded as well within the limit of tolerance of the fish, though probably above the average for the resting state.

Observations

Physiological Gas Tensions

The results on the gas contents and tensions in circulating blood are presented first, since they indicate the part of the CO_2 dissociation curves of greatest physiological importance. Table I gives the CO_2 content, percentage saturation with oxygen and estimated CO_2 tensions of samples of venous blood drawn from four species of fish. Only the trout blood could have changed significantly between the time of drawing the blood and the time of analysis. If maximum corrections for metabolism are applied to the results on trout blood, the CO_2 tension would be about

Table I

Venous blood gases

Trout	Temperature	CO_2	O_2	P CO_2
	°C.	v.p.c.	per cent sat.	mm. Hg
B	22	21.0	0	9
C	22	24.8	3	10
D	15	19.4	0	8
E	15	22.8	0	10
Carp				
J	10	36.4	47	5
G	15	31.2	30	10
H	15	28.6	18	7
Sucker				
A	8	47.8	37	9
B	8	36.3	26	7
Catfish				
A	8	21.4	62	8

1 mm. lower, the CO_2 contents about 2 vols. p.c. lower and the oxygen contents about 2 vols. p.c. higher.

No measurements were made on the aerated blood from the gills, but a rough estimate of the CO_2 tension in aerated blood can be made in the following manner. Trout *D* in Table I is favorable for purposes of illustration, because it had practically the same O_2 and CO_2 capacity as the composite blood *F* in Fig. 1. If the blood of trout *D* were fully oxygenated, it would gain about 11 vols. p.c. of 0. Assuming an R.Q. of unity, the CO_2 lost would be 11 vols. p.c., leaving a CO_2 content of about 8 vols. p.c. A CO_2 content of 8 vols. p.c. corresponds on the curve for oxygenated trout blood in Fig. 1 to a CO_2 tension of about 3.5 mm. Similar calculations for all the bloods in Table I gave estimated

CO_2 tensions of 3–5 mm. for fully oxygenated "arterial" blood. These figures indicate that the most important range of physiological CO_2 pressure is between 3 and 10 mm. Hg in all four species. It is a matter of considerable interest that the physiological range is so similar in fish representing the extremes of variation in the effect of CO_2 on the combination of oxygen in the blood. It may be concluded that the consequences of differences in sensitivity to CO_2 are not evaded to any extent, as might conceivably be the case if different species maintained themselves at significantly different CO_2 pressures.

Another implication of the foregoing results is that CO_2 tensions above 10 mm. could only be attained in any of these fish under condi-

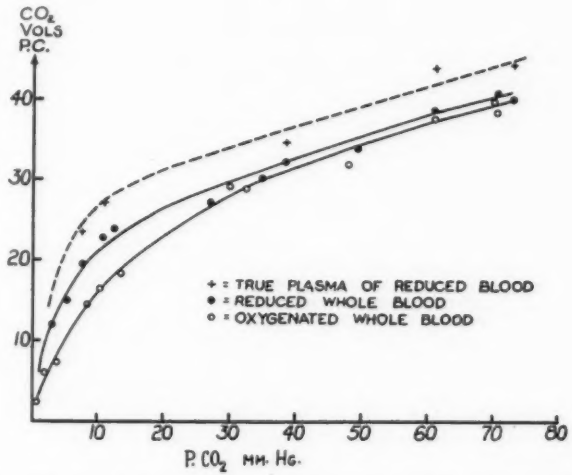

FIG. 1. CO_2 dissociation curves of pooled blood of rainbow trout at 15° C.

tions of oxygen debt. Higher tensions might occur, however, in certain tissues as a result of acid production locally. Consequently the rather low CO_2 tensions found in mixed venous blood need not be regarded as the maximal CO_2 tensions which may operate in the production of gases in the swim-bladder in deep water.

CO_2 Dissociation Curves

Figure 1 shows composite curves of two batches of blood each from twelve rainbow trout. They resemble the curves obtained by Root (1931) and Root and Irving (1940) on various marine fish in the con-

vergence of the curves for oxygenated and reduced blood at the higher pressures of CO_2. It can be seen in Table II that the oxygenated blood is not fully oxygenated at the higher pressures of CO_2 even though in some cases it was exposed to pressures of oxygen as high as 670 mm. Hg.

The apparent disappearance of the effect of oxygenation on CO_2 capacity (Haldane effect) at the higher pressures of CO_2 can be due only in part to incomplete oxygenation of the oxygenated blood because even at the high pressures it is still half saturated with O_2.

In Fig. 2 are shown the CO_2 dissociation curves of carp blood. Two sets of curves are given representing the range of variation found in six batches of carp blood. The greater variation shown by the carp may be due in part to the use of fewer fish for each batch of blood (the carp

TABLE II

Complete data on blood of trout F at 15°C. To calculate combined O_2 (Hb O_2) the Bunsen solubility coefficient of oxygen in the blood at 15° is assumed to be 0.036. The meaning of r CO_2 and r Cl is explained in the text.

P CO_2	PO_2	CO_2 content		Hb O_2	r CO_2	Cell vol.	Chloride		r Cl	Water content	
		Whole blood	Plasma				Whole blood	Plasma		Whole blood	Plasma
mm. Hg	*mm. Hg*	*v.p.v.*	*v.p.c.*	*v.p.c.*		*per cent*	*m. eq./l.*	*m. eq./l.*		*g./100 g.*	*g./100 g.*
2.2	Air	6.1	8.0	9.9	0.33	32.8	—	140.0	0.60	86.0	94.5
2.7	0	11.9	13.8	0.0	0.71	33.0	116	139.5	0.61	86.0	94.6
10.7	124	16.3	18.0	7.3	0.90	35.3	118	140.0	0.65	86.5	95.5
12.5	0	23.8	26.4	0.0	0.95	47.0	117	136.0	0.81	85.9	94.3
32.7	168	28.6	31.5	5.0	0.94	43.0	116	138.0	0.78	86.0	94.9
38.5	0	31.8	34.2	0.0	1.02	44.0	117	139.5	0.77	85.8	94.5
61.0	675	37.3	40.6	5.5	0.94	37.4	116	139.0	0.68	85.3	94.6
70.5	0	40.4	44.0	0.0	0.98	43.8	116	138.0	0.77	84.8	95.0

being larger fish), and consequently less averaging out of individual variations. The curves for carp differ from those of the trout in two important respects. Firstly they are higher, indicating a higher pH at a given pressure of CO_2. Secondly the curves of oxygenated and reduced bloods are widely separated and show only a slight tendency to converge at higher CO_2 pressures.

The higher CO_2 capacity of carp blood indicates a higher pH which must be attributed to the regulation of the acid-base balance of the fish as a whole at a more alkaline level. The CO_2 dissociation curves of true plasma in both species lie above the curves for whole blood, in this respect resembling mammalian blood rather than dogfish blood (Ferguson, Horvath and Pappenheimer, 1938). They indicate a higher concentration of CO_2 in the plasma than in cells at the same pressure of

CO_2. The distribution of CO_2 between cells and plasma is more precisely indicated in Table II in the column headed r CO_2.

Distribution of Electrolytes

The distribution ratio r CO_2 is the concentration of combined CO_2 per gram of cell water divided by the combined CO_2 per gram of plasma water. Combined CO_2 is calculated by subtraction of the physically dissolved CO_2 from the total CO_2, using the factors 0.125 and 0.105 for plasma and cells respectively, which multiplied by the PCO_2 in mm. Hg give the concentrations of dissolved CO_2 in volumes per cent. RCl is

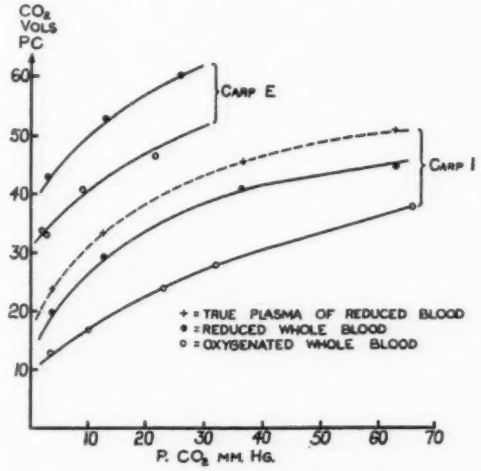

FIG. 2. CO_2 dissociation curves of carp blood at 15° C. Of six batches of pooled carp blood, E had the highest CO_2 capacity and I the lowest.

the analogous distribution ratio for chlorides. Little weight should be given to individual values of the distribution ratios because of the large number of possible errors that are introduced in their computation. On the average, however, it is evident that in carp blood the values of r CO_2 do not tend to exceed those of r Cl as they do in mammalian blood where the higher value of r CO_2 is probably due mostly to the presence of carbamino compounds of CO_2 with hemoglobin (Roughton, 1935). In the trout blood the values of r CO_2 do tend to exceed somewhat the values of r Cl. Thus the distribution ratios provide no evidence for the presence of carbamino compounds in the carp erythrocytes, but would be consistent with the presence of a small amount in trout cells. They

are consistent with the hypothesis that the combined CO_2 in red cells is largely in the form of bicarbonate and that the bicarbonate and the chloride are partitioned between the red cells and plasma according to a Donnan distribution. The figures on plasma chloride of carp blood in Table III give further evidence of a Donnan equilibrium with chloride and bicarbonate ions diffusible. The plasma chloride decreases regularly (except for one figure) with each increase in plasma CO_2 consistent with a migration of chloride into the cells with increasing acidity. Plasma chloride in trout blood also shows a decrease with increasing CO_2, but in a very irregular fashion which, however, acquires more meaning when considered with the changes in packed cell volume.

TABLE III

Complete data on blood of carp I at 15°C.

P CO_2	PO_2	CO_2 content		Hb O_2	$r\ CO_2$	Cell vol.	Chloride		r Cl	Water content	
		Whole blood	Plasma				Whole blood	Plasma		Whole blood	Plasma
mm. Hg	*mm. Hg*	*v.p.c.*	*v.p.c.*	*v.p.c.*		*per cent*	*m. eq./l.*	*m. eq./l.*		*g./100 g.*	*g./100 g.*
3.4	158	12.9	15.2	12.3	0.77	39.6	124.5	146.0	0.94	82.5	94.8
3.7	2.3	19.8	23.8	1.8	0.77	41.2	125.0	144.0	0.88	83.9	94.8
10.5	148	17.0	21.2	12.7	0.61	38.8	125.0	146.5	0.90	84.1	95.1
12.6	0	29.3	33.7	0.4	0.88	40.6	125.5	142.5	0.91	83.5	94.6
32.2	145	27.9	31.6	10.8	0.90	39.5	125.5	144.5	0.85	83.2	95.0
36.6	0	41.1	45.9	0	0.95	40.2	126.0	141.5	0.94	83.4	94.5
66.2	224	38.1	42.2	10.8	0.98	40.9	125.0	142.0	0.92	83.6	94.4
63.2	0	45.0	51.3	0	0.88	41.2	124.5	140.5	0.95	82.7	95.2

Cell Volumes

In Fig. 3 the packed cell volumes of carp and trout blood are plotted against pressure of CO_2. In carp blood the cell volume decreases slightly with the first increment of CO_2 pressure, but with further increase in P CO_2 the volume increases as in mammalian blood, and by about the same amount for each increment of CO_2 combined, namely by about 5 p.c. of their volume for an increase of 10 m.eq. per liter of combined CO_2. The cells of T.J.F. (Henderson, 1928) increased by 6 per cent for an increase of 10 m.eq. of CO_2 combined.

The volume changes for trout blood shown in Fig. 3 are the composite data from three batches of blood. The trout cells show greater changes in volume than do the carp cells. They reach their maximum size between 10–20 mm. Hg of CO_2 pressure. The reduced cells show a much greater increase in volume, but at pressures above 20 mm. Hg they decrease again with increasing P CO_2. It can be seen in Table II

that the largest cell volume corresponds with the lowest concentration of chloride in the plasma, suggesting that a migration of chloride from the plasma, presumably into the cells, has occurred. The extent of this migration is depicted in Fig. 4, where the chloride content in the plasma of a liter of blood is plotted against the bicarbonate content of the plasma of a liter of blood. The contents are calculated by multiplying the concentrations per liter of plasma by the fraction of the whole blood volume which is plasma. In carp blood it appears that for each increase of 1 eq. of bicarbonate in the plasma about 0.75 eq. of chloride enters the cells. This ratio is approximately that for mammalian blood (Van

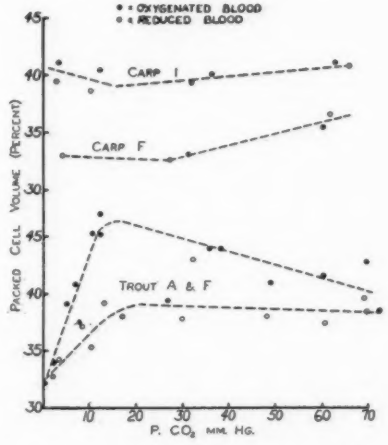

FIG. 3. Packed cell volumes in carp and trout blood are plotted against the pressure of CO_2. The large changes in cell volume in trout blood are in striking contrast to the small changes in carp blood.

Slyke, 1921). In trout blood, however, the loss of chloride from the plasma at the maximum cell volume greatly exceeds the increase in CO_2 in the plasma. This suggests that the excessive cell volumes and excessive chloride shift may be due to the production in the blood of an acid other than CO_2. It is immaterial where this acid is produced, but it is necessary to postulate that it is diffusible through the red cell membrane. It is also necessary to postulate that it is produced by a reversible reaction and that the equilibrium point is determined by the tension of oxygen and of CO_2 in the blood. The optimum conditions for its formation would presumably be at low oxygen tensions and a CO_2 tension between 10 and 20 mm. To explain the cell volume changes by

this production of acid alone would require the formation of about 30 m.eq. per liter of acid other than CO_2. Such an hypothesis certainly deserves the utmost skepticism till the changes in cell volume can be checked by a method other than centrifuging, but it does receive some support of a qualitative nature from another and independent consideration, namely the effect of oxygenation on CO_2 capacity.

Oxygenation and CO_2 Capacity

When the effect of oxygenation on CO_2 capacity is expressed as $-\Delta BHCO_3/\Delta O_2$ at constant plasma pH the maximum values for this

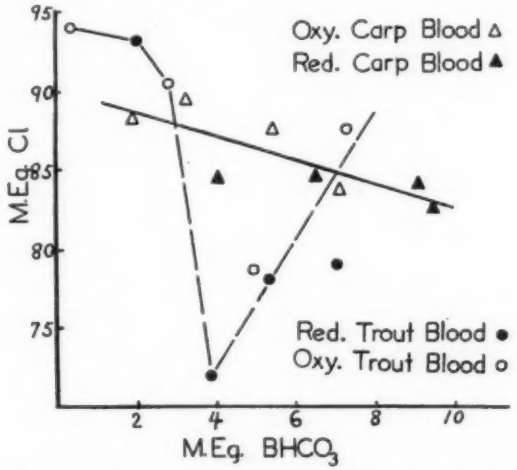

FIG. 4. The chloride in the plasma of a liter of blood is plotted against the bicarbonate in the same volume to illustrate the magnitude of the migration of chloride into the cells. In carp blood $\Delta BHCO_3/\Delta Cl = -.75$ as in mammalian blood, but in trout blood the ratio is much greater than unity, suggesting that an acid other than CO_2 has been neutralized in the cells and diffused into the plasma in exchange for chloride ions.

ratio are found at a pH of about 7.3 in both bloods. The maximum value in each is about 1.2. This value is higher than any of those in other species compiled by Redfield (1933), as it should be, in accordance with the principle that the greater the effect of acid on the combination of oxygen the greater should be the effect of oxygenation on the dissociation of hemoglobin as an acid. But the fact that the value is the same in trout and carp blood is apparently inconsistent with the principle. If, however, the hypothesis of "extra acid" production in trout

blood is correct, the apparent inconsistency would be only apparent, for if an acid other than CO_2 were produced in the blood at low pressures of oxygen, $\Delta BHCO_3$ would not be a complete measure of the change in base combined with hemoglobin, and hence would give too low an estimate of the effect of oxygenation on the acid dissociation of the hemoglobin in trout blood.

Buffer Power

The buffer power (β) of whole blood is often represented by the ratio $-\Delta BHCO_3/\Delta pH_s$, where $\Delta BHCO_3$ is the change in CO_2 combined in whole blood and pH_s the pH of the plasma or serum. This procedure will be erroneous in trout blood if the hypothesis of the extra acid production is correct. It is interesting, however, to make the cal-

TABLE IV

Buffer power of reduced bloods.

	Range of plasma pH	Range of P CO_2	Buffer power (β)
Carp 15°	8.11–7.36	2–20	1.6
	7.36–6.96	20–60	1.4
Trout 15°	7.90–7.23	2–20	2.3
	7.23–6.85	20–60	1.7
*Human 38°	7.5–7.2	35–90	2.3

* Peters and Van Slyke, Quantitative Clinical Chemistry, Vol. I.
$\beta = -\Delta BHCO_3/\Delta pH/Hb$. Concentrations are in milliequivalents per liter.

culation in the conventional manner and then to consider what change in conclusion would be necessary if the hypothesis of extra acid production were correct. The calculated buffer powers of carp, trout and human blood adjusted to equal concentrations of hemoglobin are given in Table IV. Figures are given for reduced blood only, since fully oxygenated trout blood cannot be obtained over a large enough range of pH. Carp blood has about the same buffer power over the range studied, but trout blood shows a greater buffer power over the more physiological range of P CO_2 of 2–20 mm., where it is equal to the buffer power of human hemoglobin. Over the higher range of CO_2 pressure the buffer power of trout hemoglobin is less, though still higher than that of carp blood. The trout blood shows a concentration of its buffer power in the physiological range, a characteristic which may be attributed tentatively to closer grouping of the dissociation constants of

the acid (or basic) groups in this range. If the anomalous increase and decrease in cell volume in trout blood represents increase and decrease in extra acid, the true buffer power over the range 2–20 mm. would be even greater, and even less over the range 20–60 mm. In other words, the tendency for the buffer power to be concentrated in a narrow range in the trout blood would be even more striking.

Plasma pH

Values of plasma pH at CO_2 pressures of 2, 20 and 60 mm. Hg calculated from the smoothed data of the dissociation curves, assuming a pK_1' of 6.2 are shown in Table V. The changes in plasma pH may be taken as paralleling fairly closely the changes in cellular pH. To calculate these separately would merely introduce the uncertainties of arbitrary values for pK_1' in cells.

The main points of interest are: (1) an increase of P CO_2 from 2 mm. to 20 mm. produces a greater change in pH in carp blood than

TABLE V

pH of true plasma of oxygenated blood calculated from the data of Tables II and III.

P CO_2	Carp	Trout
mm. Hg		
2	7.91	7.66
20	7.23	7.15
60	6.84	6.84

in trout blood; (2) at P CO_2 of 60 mm. the carp blood is as acid as the trout blood. In other words, the loss in oxygen capacity in trout blood cannot be attributed either to a greater change in acidity for a given increase in P CO_2 or even to a higher absolute acidity at the higher pressures of CO_2. However, the lower acidity in carp blood at lower pressures of CO_2 must be held partly responsible for the higher affinity of the blood for oxygen in the absence of CO_2.

DISCUSSION

The results as a whole indicate that the great differences in the effect of CO_2 on the combination of oxygen in these two bloods and their affinity for oxygen may be due to adaptations at three levels of physiological organization. These seem to be: (1) specific differences in the hemoglobin molecule; (2) differences in the environment provided for the hemoglobin by the erythrocyte; (3) differences in the acid-base regulation of the fish as a whole. It seems likely that differences in the

hemoglobin molecules will prove to be the most important element in the total adaptation, although no comparison of the two hemoglobins in solution has yet been made. Until such a comparison is made, it cannot be said that differences in the erythrocytes may not be equally important. Certainly one of the most striking differences between the bloods has been the behavior of the cell volumes. The effect of the intracellular environment on the affinity for oxygen is marked, even among mammals, and varies from species to species (Hill and Wolwekamp, 1936) in a manner as yet inexplicable. In fish blood the effect of hemolysis on affinity for oxygen is also large and cannot be explained by changes in acidity alone (Root and Irving, 1940). The anomalous behavior in trout blood of cell volumes, plasma chlorides and effect of oxygenation on CO_2 capacity could all be explained, at least in part, by the production (by a kind of Pasteur reaction) of acid at low tensions of oxygen and an optimal tension of CO_2. The production of extra acid cannot, however, explain the loss of oxygen capacity or the convergence of the CO_2 dissociation curves of oxygenated and reduced blood. At most it could only be a mechanism augmenting the effect of CO_2 in maintaining a high tension of oxygen at low contents of oxygen in the blood of trout.

Summary

The venous blood from four species of freshwater fish, rainbow trout (*Salmo gairdnerii* Richardson), carp (*Cyprinus carpio* Linnaeus), bullhead (*Ameiurus nebulosus* Le Sueur) and sucker (*Catostomus commersonnii* Lacepède) was analyzed and an estimate made of the probable range of physiological CO_2 tension.

A detailed study was made of CO_2 transport in the blood of two of these species, the rainbow trout and the carp, which differ greatly in the effect of CO_2 on the combination of oxygen in the blood. They differ too, in their systems of CO_2 transport. A curious feature of trout blood is the great change in packed cell volume with changes in the pressure of O_2 and CO_2. An hypothesis is presented to explain in part these anomalous changes in cell volume and other characteristics of the trout blood. Carp blood shows less differentiation from general vertebrate characteristics.

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THE DEVELOPMENT OF THE ASCIDIAN EGG CENTRIFUGED BEFORE FERTILIZATION

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The development of centrifuged eggs of ascidians has been studied by Duesberg (1926) and by Conklin (1931). In *Styela* and *Ciona* Conklin found that when the eggs were centrifuged after fertilization, three substances, namely the mitochondria, the hyaloplasm, and the yolk may be displaced from their normal positions into three zones. If these substances were so held until the beginning of cleavage, they were distributed abnormally to the blastomeres. The tissues or organs of the embryos derived from these eggs were also dislocated.

The unfertilized eggs of *Styela* and *Ciona* also were centrifuged by Conklin. He noted that under strong centrifuging the spermatozoön frequently did not enter the egg at all. No description of the development of these eggs was given.

Recently Dalcq (1932, 1935, 1938) has fragmented unfertilized eggs of *Ascidiella scabra* into two parts and then fertilized them. He found that meridional halves of egg fragments may give rise either to apparently normal and symmetrical larvae, or to lateral half larvae; while larvae obtained from latitudinal halves may be deficient in one or more kinds of tissues according to the level of the cut. These experiments indicate that the unfertilized egg already possesses germinal localizations; but at the same time it has a great capacity for regulation, as has been demonstrated by Tung (1934) for the fertilized egg.

To gain further light on the organization of the unfertilized egg of the ascidian and to learn whether, with sufficient force, the organ-forming substances might be dislocated, we have made a study of the development of *Ciona* eggs strongly centrifuged before fertilization. The experiments on which this investigation is based were performed in the summer and autumn of 1936.

[1] This work was done while the senior author was on the tenure of a grant from the Board of Trustees for the administration of the indemnity funds remitted by the British Government for which he wishes to express his gratitude.

Material and Methods

The observations recorded here were all made on eggs of *Ciona intestinalis* obtained in the vicinity of Tsingtao. During the months from June to October the gonoducts of *Ciona* are usually full of ripe eggs and spermatozoa. The eggs were obtained free from sperm by removing the integuments and carefully opening the oviduct which is near the surface. The eggs were then removed from the oviduct with a pipette and transferred to a glass dish containing fresh sea water. A part of the eggs was used for centrifuging, leaving the remaining eggs in the dish as a control.

The eggs to be centrifuged were placed in glass tubes with 10 cc. sea water and rotated for ten minutes to one hour and fifty minutes at a speed ranging from 2000 r.p.m. to 3800 r.p.m. These speeds represented centrifugal forces of about 716 × gravity and 2585 × gravity. In order to prevent eggs from rotating during centrifugation, capillary tubes were used as by Conklin. After centrifuging, the eggs were immediately removed from the capillary tubes and fertilized by sperm of another animal.

Individual eggs showing an abnormal distribution of oöplasmic substances to the first two or four blastomeres were picked out, sketched, and placed in separate dishes of fresh sea water in order to study in detail the location of various tissues in later development.

The larger part of the eggs and embryos was fixed in Bouin's fluid and double-embedded in agar-paraffin (Chatton, 1927). Sections were cut at 7 μ and stained with iron-haematoxylin, eosin, and light green. Some of the material was fixed in Flemming's solution and mounted in toto in order to determine the location of mitochondria.

Results

Stratification of the Egg

The degree of the stratification of oöplasmic substances of the unfertilized egg varies with the rate and duration of centrifugation. In strongly centrifuged eggs three zones can be distinguished. These are: (1) an alveolar or light zone at the centripetal pole; (2) a middle clear zone containing the nuclear elements; and (3) a lower, heavy zone of yolk. In eggs fixed in Flemming's solution and mounted in toto, the light zone consists exclusively of large black, densely packed granules. On the basis of the staining reaction, it corresponds obviously to the "mitochondria" zone of Duesberg. Sections of the eggs show that this zone is composed of an alveolar substance in which are embedded

a large number of blue-black granules; these are taken to be mitochondria (Fig. 1).

Under prolonged centrifuging, a fourth zone appears at the centrifugal pole. Figure 2 shows an egg in which the four zones are

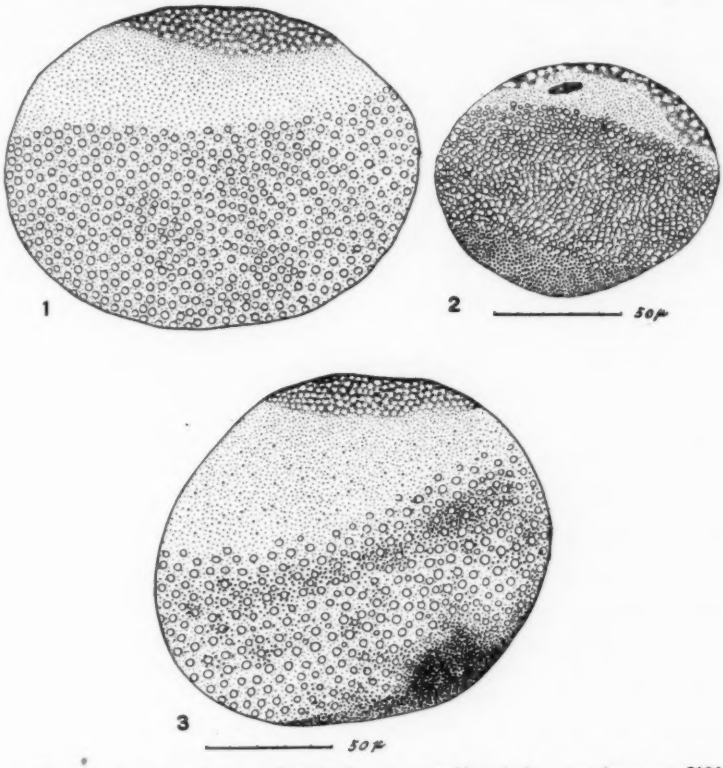


FIG. 1. Section of an unfertilized egg, centrifuged for one hour at 2193 times gravity, and fixed ten minutes later, showing a stratification of the egg contents into three zones.

FIG. 2. Section of an unfertilized egg centrifuged for one hour at 2193 times gravity and fixed ten minutes later, showing the fourth zone at the centrifugal pole.

FIG. 3. Section of an unfertilized egg, centrifuged for one hour at 2193 times gravity and fixed fifteen minutes later. The stratification of the centrifugal zone is not complete.

clearly separated. The centrifugal zone contains blue-black granules, while in the centripetal zone the number of the same kind of granules is greatly reduced. Figure 3 shows an egg with incomplete stratification of the fourth zone. In this case a part of the granules remains

scattered throughout the clear and yolk zones and especially in the latter. A comparison of the eggs illustrated in Figs. 1, 2, and 3 leaves no doubt that the granules of the centrifugal zone are separated out from the alveolar substance of the centripetal zone in which they were formerly embedded.

Conklin described a fourth zone between the hyaline and yolk zones and concluded that ". . . It (the substance of the zone), rather than the mitochondria, is the myoplasm or formative substance for the future muscles." Our conclusions are that it is the alveolar substance of the centripetal zone that is the formative substance of the muscles; the alveolar substance is similar to the cytoplasm of muscle cells of the young embryo both in structure and in staining reaction, whereas the granules

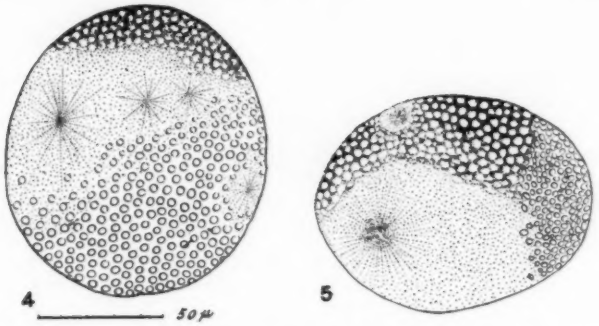


FIG. 4. Section of a polyspermic egg centrifuged for one hour at 2193 times gravity and fixed fourteen minutes after fertilization. Note sperm asters in clear and yolk zones.

FIG. 5. Section of a polyspermic egg centrifuged for one hour at 2193 times gravity and fixed fourteen minutes after fertilization. Note sperm asters in clear and alveolar zones.

of the centrifugal zone are the mitochondria since in normal fertilized eggs similar granules are found in the lower hemisphere at which the sperm enters. In *Physa heterostropha,* Clement (1938) has recently shown that the mitochondria which stratify between the clear and yellow zones and are therefore heavier than the clear protoplasm are the last to be segregated under centrifugal forces. In these respects our observations on *Ciona* agree with those of Clement on *Physa.*

Immediately after centrifuging, the eggs were cross-fertilized with fresh sperm. As a check, in each experiment, the control eggs left unfertilized in the original dish were examined. As has recently been reported by Morgan (1938), cleavages were rare, showing that self-fertilization occurs rarely in *Ciona.* Conklin found that in eggs which

had been strongly centrifuged before fertilization, the spermatozoön frequently did not enter at all. He attributed the failure of fertilization to the compactness of the yolk at the vegetative pole which blocked the entrance of the spermatozoön. In our experiments, though we have no detailed record, the percentage of fertilization in centrifuged eggs is generally not very low in comparison with normally fertilized eggs.

In sections it appears that the spermatozoön may penetrate the egg in the clear zone or between this zone and the yolk; for the most part it enters the yolk zone (Fig. 8). This indicates that the compactness of the yolk is not a factor in blocking the entrance of the spermatozoön. In polyspermic eggs the sperm asters are found in almost any part of the egg, even in the alveolar substance of the centripetal zone. Figures 4 and 5 show two such eggs that had been centrifuged for one hour at 2193 $\times$ gr. and were fixed fourteen minutes after fertilization. The entrance points of the sperm are suggested by the positions of the asters.

The first maturation spindle of the strongly centrifuged eggs always lies in the clear zone. After fertilization it moves to the periphery of the zone where the polar bodies are given off. In some polyspermic eggs, when a sperm-nucleus has already migrated to the center of the egg, the first maturation spindle still rests in the middle of the clear zone. In such cases probably no polar bodies will be given off; they will be retained in the egg.

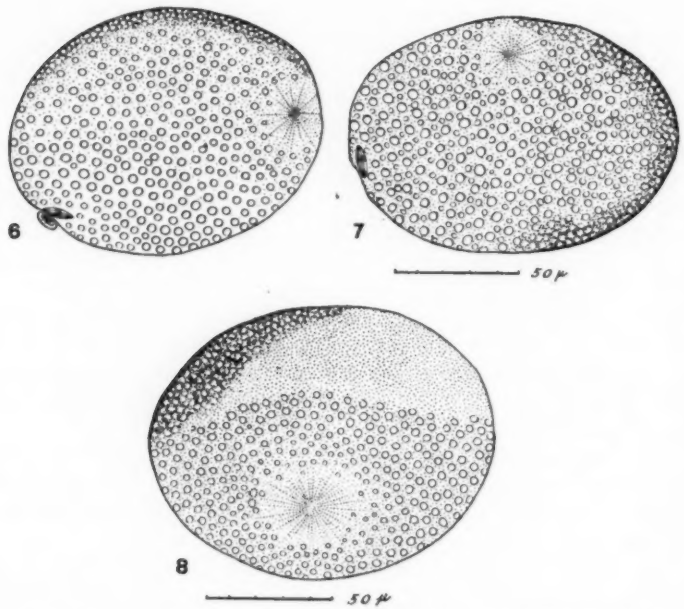
The entrance point of the spermatozoön may be found at any plane with respect to the position of polar bodies. This is evident in Figs. 6 and 7, which represent two eggs centrifuged for ten minutes and fixed one hour after fertilization.

In the normal egg a protoplasmic movement usually takes place immediately after the entrance of the sperm. Such movements probably also occur in the weakly centrifuged eggs with incomplete stratification of oöplasmic substances, since after fertilization the different substances of such eggs return to their normal positions. In strongly centrifuged eggs the movement of the oöplasm is less marked. This is well demonstrated in Fig. 8. This egg was centrifuged in a capillary tube for one hour and twenty-four minutes at 2193 $\times$ gr., and fixed forty minutes after fertilization. The three principal zones are still quite clear. The sperm aster is found in the yolk zone surrounded by a clear substance and no mitochondria. It appears that the free movement of the substances is impeded by the stratification.

Cleavage

As a check, in every experiment some of the control eggs were fertilized at the same time as the centrifuged eggs. The first cleavage

of the eggs of both sets occurred at the same time, regardless of the rate and duration of centrifuging. It took place usually in about one hour and a half after insemination, much later than the records of other authors. Such delayed cleavage may be due to the low temperature of the sea water at Tsingtao.

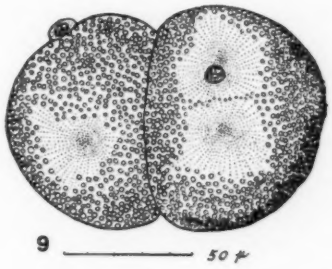


FIGS. 6 and 7. Sections of eggs centrifuged for ten minutes at 2193 times gravity and fixed one hour after fertilization, showing the positions of the sperm aster, the polar body (Fig. 6) or second maturation spindle and the mitochondria crescent. There are no constant relations between them.

FIG. 8. Section of an egg centrifuged in capillary tube for one hour and twenty-four minutes and fixed forty minutes after fertilization. The sperm aster is in the yolk zone; no mitochondria surround it.

The pattern of the cleavage planes of the centrifuged eggs differed in no essential respect from those of normal eggs. The first two cleavage planes were perpendicular to each other, while the third plane was at right angles to both of the first two, resulting in the formation of four micromeres and four macromeres. However, the first two cleavage planes did not always pass through the point of attachment of the polar bodies. After prolonged centrifuging, there was a significant

percentage of eggs whose polar bodies were not at the first cleavage furrow. The deviation between them may be of any angle up to 90° (Fig. 9). Since after centrifuging there was no definite landmark to indicate the original position of the animal pole and since the orientation of the eggs during the process of centrifuging could not be determined accurately, it was impossible to ascertain whether the polar bodies were



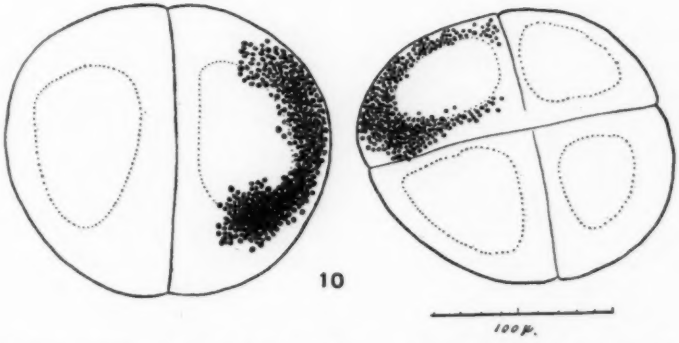


FIG. 9. Section of an egg in 2-cell stage. The polar body is not located at the cleavage furrow.

FIG. 10. Two entire eggs in 2- and 4-cell stages, showing mitochondria in one blastomere.

produced at other than their normal position, or whether the first cleavage plane passed through the original animal pole.

In most eggs the first cleavage plane appears to coincide with the axis of centrifugation. As the stratified substances are rarely equally distributed around the axis, it divided them into more or less unequal halves. However, there is a significant percentage of eggs in which the

first cleavage appeared in any plane and at any angle with respect to the stratification of substances. It may be oblique to the axis of centrifugation or perpendicular to it. In the latter case one blastomere contains only the yolk spherules and the other the clear and alveolar substances. Figure 10 shows two such eggs in the 2- and 4-cell stages respectively. They were fixed in Flemming's solution and mounted in toto. The mitochondria or alveolar substance is confined to one blastomere. Similar conditions are also shown in Fig. 9.

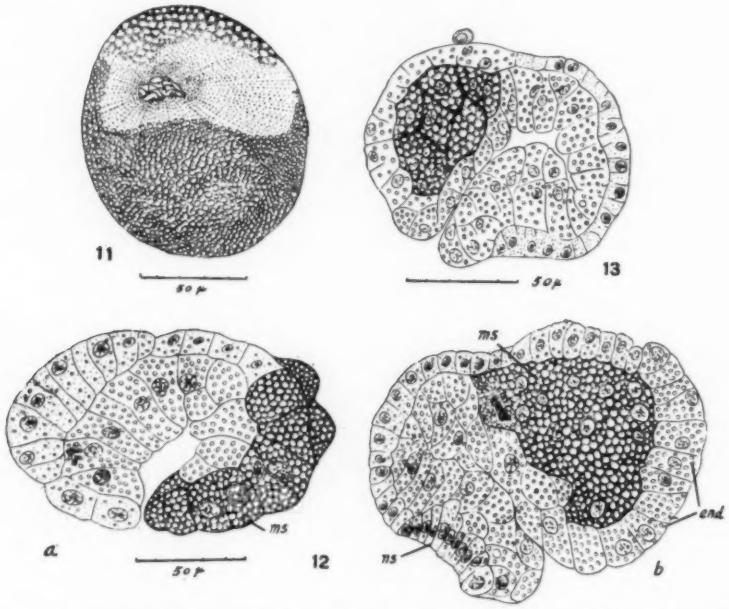


FIG. 11. Section of an egg centrifuged for one hour at 2193 times gravity. The three oöplasmic zones remain unchanged after fertilization.

FIG. 12. Section of two gastrulae, showing the abnormal positions of muscle (*a*) and endoderm (*b*) cells. *ms.*, muscle cell; *end.*, endoderm; *ns.*, neural cell.

FIG. 13. Section of a gastrula derived from egg centrifuged fifteen minutes at 2193 times gravity. The polar body is situated posterior to the middle of the ventral surface in its typical position.

It not infrequently happens that the first cleavage is quantitatively unequal; this leads to a formation of a larger macromere and a smaller micromere. Sometimes the second cleavage plane does not intersect the first at right angles, so that the four resulting blastomeres do not lie in the same plane. The cleavage pattern in such eggs as well as in those described above seems to be determined by the position of the

cleavage spindle. In cases where the three principal zones remain unchanged after fertilization, the first cleavage spindle always lies horizontally in the clear zone. In such eggs the cleavage plane coincides with the axis of centrifuging. If a slight change of the stratification takes place before the division, the position of the mitotic spindle is also changed. The subsequent cleavage will then divide the egg in any plane with respect to the axis of the centrifuging. Figure 11 shows a section of an egg in which the oöplasmic substances were stratified into three zones. The two pronuclei had come together in the center of the clear zone. The first cleavage of this egg may be expected to approximate the axis of stratification and will distribute the oöplasmic substances equally between the two blastomeres.

In many eggs the division of the cell body is suppressed while the division of centers and chromosomes continues. In such eggs are found numerous nuclei and centers, confined to the alveolar and clear zones. Such anomaly of cleavage is evidently not due to any direct effect upon the mitotic figure, for the fertilization of the eggs took place after centrifuging.

Later Development of Centrifuged Eggs

The later development of centrifuged eggs is particularly interesting since the dislocated tissues or organs can be identified in stained sections. Individual eggs showing abnormal distribution of oöplasmic substances to the first two, four, or eight blastomeres were sketched and isolated. The development of these eggs was studied. In general, it may be said that the stronger and the longer the centrifuging, the more abnormal was the subsequent development.

The gastrulation of strongly centrifuged eggs was rarely typical. Figure 12 shows two abnormal cases in which a part of the mesoderm and entoderm cells had not been invaginated. In normal development the polar bodies are situated on the anterio-ventral part of the older gastrula. In Fig. 13 a polar body is found posterio-ventrally on the gastrula, indicating that gastrulation took place independent of the position of the polar bodies.

Following prolonged centrifugation, a large number of eggs started their development but most of them never reached a stage at which they could be recognized as larvae. The embryos were so abnormal in form that it was impossible to identify their parts and organs except by a histological study. In these embryos, tissues and organs which will be described separately in the following paragraphs were usually displaced. Embryos are found composed of three types of cells, namely: (1) muscle and mesenchyme cells; (2) ectoderm and neural cells; and (3)

notochordal and endoderm cells. These types correspond to the products of the three zones (viz., alveolar substance, clear cytoplasm, and yolk) which had been stratified by the centrifugal force.

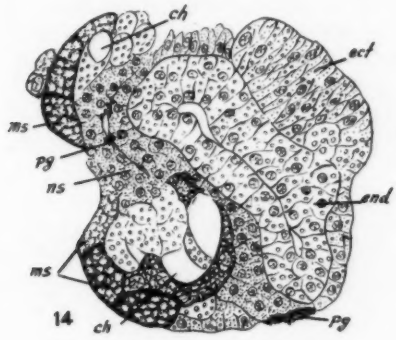
Muscle Cells and Mitochondria.—In typical embryos and larvae, the large muscle cells are arranged in the tail in three rows on each side of the notochord. Owing to their large nuclei and specific staining reaction, they are easily distinguished from other types of cells. In the abnormal larvae derived from centrifuged eggs, isolated or aggregated cells of this type may be found in the interoir or at the surface (Fig. 14). They are rarely arranged regularly even when they are found alongside of the notochord. In some cases these cells lie in the midst of the endoderm or just under the neural tissue. Figure 15 shows a section of a larva in which a part of the gut wall is formed of cells other than typical endoderm. Though one cannot be certain as to whether these are really mesoderm cells or not, the structure of the cytoplasm and the size of the nucleus are similar to those of the latter.

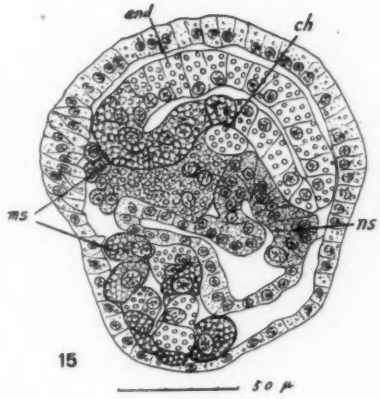
The mitochondria which are normally embedded in the cytoplasm of the muscle cells may have been driven into the regions subsequently forming ectoderm (Figs. 16 and 19*a*) or neural tissue (Fig. 15). The myofibrillae are often found in the muscle cells, but are never found in those tissues into which the mitochondria have been driven. Our observations, therefore, confirm the view of Conklin, that in *Ciona* the mitochondria do not give rise directly to myofibrillae.

Notochordal Cells.—Owing to the abundance of yolk spherules, the notochordal cells of the young embryo are very similar to those of the endoderm. In larvae, on the other hand, they are characterized by the possession of large vacuoles and can be thus recognized wherever they occur. In most abnormal larvae derived from strongly centrifuged eggs, notochordal cells did not arrange themselves to form a rod, but instead were displaced to various positions. They may be grouped together or scattered. In some cases, they are found at the surface (Fig. 17) of the larvae or just under the ectoderm (Fig. 16). In other cases they are in the midst of endoderm or surrounded by muscle cells. In such embryos no tail is formed.

The neural tissue which will be described in the next paragraph was not always associated with the notochordal cells; nor do the latter exert any influence on the differentiation of the ectoderm cells with which they are in contact. We agree, therefore, with Berrill and Conklin who have concluded that the notochord of the ascidian does not act as an organizer in the sense of Spemann.

Neural Cells and the Sensory Pigment.—The neural cells may occur in any portion of these abnormal larvae. Sometimes they formed a





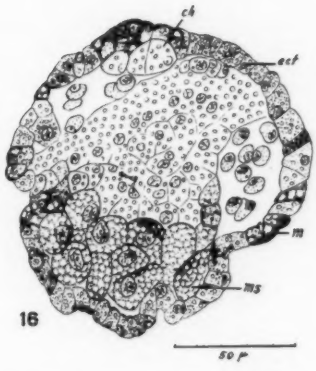


FIG. 14. Section of an abnormal larva derived from an egg centrifuged for thirty minutes at 2193 times gravity and fixed twenty-eight hours after fertilization. *ms.*, muscle cell; *ch.*, notochord; *pg.*, pigment spot; *ns.*, neural cell; *ect.*, ectoderm; *end.*, endoderm.

FIG. 15. Section of an abnormal larva derived from an egg centrifuged for one hour and thirty minutes at 2193 times gravity and fixed twenty-four hours after fertilization. A part of the gut wall contains alveolar substance. *ms.*, muscle cell; *ns.*, neural cell; *end.*, endoderm; *ch.*, notochord.

FIG. 16. Section of a larva from an egg centrifuged for one hour and fifty minutes, showing the mitochondria in the ectoderm and neural cells. *m.*, mitochondria; *ms.*, muscle cell; *ch.*, notochord.

plate on the surface; at other times they were grouped forming a mass of neural tissue. Usually, however, they lined a large irregular cavity extending into the interior of the larva. In larvae having well-developed tails, such a neural cavity is always found at the junction of the trunk

and the tail. The latter often turned dorsally, giving the larvae a curvature in an atypical direction. Figure 18 shows a larva of this kind. The neural cavity with two spots of sensory pigment is surrounded by the curved tail on one side. Conklin has suggested that the elongation of the neural plate and tube depends upon the normal elongation of the notochord. Our observations, however, do not lead to this conclusion.

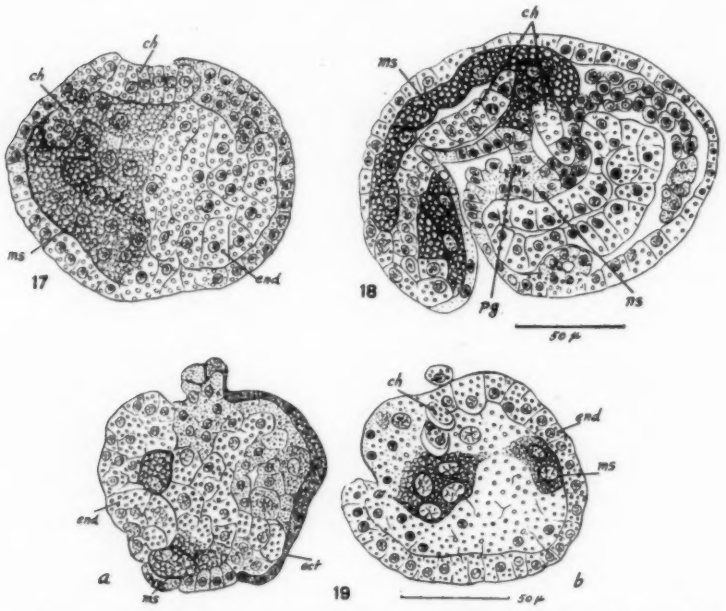


FIG. 17. Section of a larva from an egg centrifuged in capillary tube for forty minutes at 2193 times gravity. *ch.*, notochord; *ms.*, muscle cell; *end.*, endoderm.

FIG. 18. Sagittal section of a larva derived from an egg centrifuged one hour and thirty minutes at 2193 times gravity, showing the inverse body curvature. *ch.*, notochord; *ns.*, neural tissue; *pg.*, pigment spot; *ms.*, muscle cell.

FIG. 19. *a.* Section of a larva from an egg centrifuged in a capillary tube for fifty minutes at 2585 times gravity. The left yolk-filled cells are endoderm and the right mitochondria-filled cells are ectoderm.

b. Section of a larva from an egg centrifuged for fifteen minutes at 2193 times gravity. The yolk-filled cells form a superficial epithelium. *ch.*, notochord; *ect.*, ectoderm; *end.*, endoderm; *ms.*, muscle cell.

The sense organs are also structures of interest. In typical larvae there are two sense organs, the eye and the otocyst, with their pigments situated on the wall of the brain vesicle. Typical organs are rarely formed in abnormal larvae; instead, pigment spots are found in unexpected places. The number of spots varies from none to four. Their

locations may be near together or widely separated (Fig. 14). In some cases they project from the surface of the larva and in others they are embedded in the neural cells. However, they are always associated with neural tissue and notochordal cells.

Endoderm and Ectoderm Cells.—The histological characteristic of endoderm is its large yolk-filled cells. In these abnormal embryos, however, it is not easily identified, for the yolk spherules are also found in other cells of the embryo. In such a case as that shown in Fig. 19*A* there is no doubt but that the superficial cells of the left side are endodermal and those of the right side are ectodermal. In the case shown in Fig. 19*B*, on the other hand, one cannot be certain as to whether the yolk-filled surface epithelium labeled "end" is true endoderm or is ectoderm. Tung (1934), in experiments on the combination of blastomeres in *Ascidiella scabra*, has shown that the development of ectoderm and endoderm is not strictly mosaic. When the endodermal cells lie at the surface of the embryo, they may form a regular superficial epithelium. Similarly, if ectodermal cells come to lie in the interior, they may transform into large irregular endoderm-like cells. Such a regulative capacity of ectoderm and endoderm seems to exist also in the tissues of *Ciona* embryos.

In these larvae having displaced organs, the endodermal cells may be found in many abnormal locations. The fact that they are not always associated with neural tissue and pigmented sensory spots indicates that they do not serve as organizers.

Papillae.—The normal embryo possesses three papillae; two are paired and are situated dorso-laterally on each side; one is median and ventral. Papillae are also observed in some of the abnormal larvae. Their number varies from none to three. It is of interest to note that in spite of the dislocation of other organs, the development of papillae is always in relation to the endoderm. Without exception in all 49 cases observed, the papillae were formed in ectoderm underlain by endoderm. Tung (1934), on the basis of experiments on *Ascidiella scabra* in which the four animal blastomeres were rotated through 180° over the four vegetal blastomeres, has suggested that papillae are evoked in the ectoderm by underlying endoderm. The present observation furnishes further evidence in support of that hypothesis.

From the above descriptions it is obvious that the different tissues and organs of the many larvae derived from eggs centrifuged before fertilization, are out of their normal positions. Such placement of tissues is undoubtedly the result of the placement of the oöplasmic substances from which the tissues are derived. In some cases the quantity of certain tissues seems to be reduced. We have not, however, made a count of the cellular components of these tissues.

Discussion

Conklin (1905 *a* and *b*), in his classical studies on the development of ascidians, described four different kinds of oöplasmic substances in the fertilized egg, namely the ectoplasm, endoplasm, mesoplasm, and chorda-neuroplasm. From these are derived the ectoderm, endoderm, mesoderm, and the notochordal and neural tissue respectively. According to Conklin (1905*c*), the development of these tissues or organs is strictly mosaic. When the eggs were centrifuged after fertilization, tissues of the larvae which developed were displaced from their normal positions (1931). Such displacements of tissues were attributed to corresponding displacements of oöplasmic substance by means of centrifugal force.

As has been shown in the present experiments, the stratification of oöplasmic substances of eggs centrifuged prior to fertilization and the displacement of the tissues or organs of the larvae derived from these eggs are in general similar to those obtained from eggs centrifuged after fertilization. It is reasonable, therefore, to conclude that the different kinds of oöplasmic substance described by Conklin in fertilized eggs have already existed in the egg before fertilization. This conclusion agrees with that of Dalcq (1932, 1935, 1938), who likewise concluded that organ-forming substances are already differentiated in the unfertilized eggs. Conklin has suggested that the unfertilized egg of *Cynthia* possesses a bilateral symmetry. If so, it is undoubtedly due to the bilateral arrangement of these oöplasmic substances.

In recent years the idea that the ascidian egg is strictly mosaic has been called in question, though it is still insisted upon by Berrill (1932) and by Cohen and Berrill (1936). Schmidt (1931) observed three papillae in larvae derived from one-half blastomeres of *Ciona* and *Phylusia* eggs. Reverberi (1931) has obtained normal larvae from fragments of fertilized *Ciona* eggs. Tung (1934) has found in *Ascidiella scabra* that the endoderm and ectoderm are relatively equipotential and that the development of papillae and sensory pigments is always associated with endoderm and notochord respectively. Recently, Von Ubisch (1938) has described a normal embryo of *Ascidiella aspersa* produced by fused eggs. All these investigations show that after fertilization the ascidian egg has a considerable capacity for regulation.

Dalcq has reported a certain degree of regulation in the unfertilized egg of *Ascidiella scabra*. A fragment of the egg may give rise to an embryo very similar to the control. The number of cells of muscles or notochord of a pair of embryos derived from two fragments of one egg may be double the total number of a normal embryo. Moreover, in the experiments reported here, we have demonstrated that the ectoderm and

endoderm constitute relatively equipotential systems and that the papillae and sensory pigment do not appear to be self-differentiating organs. These facts plainly show that the eggs of *Ascidiella* and *Ciona* are not strictly mosaic prior to fertilization.

The recent work of Rose (1939) shows that the anterior vegetal region of the *Styela* egg is the cerebral inductor. This region contains materials essential to the differentiation of endoderm, notochord and a part of the neural tissue. In the larvae with displaced tissues or organs we have not found any typical relations between the cerebral vesicle and either notochord or endoderm. In short, there is no evidence in the present experiments to indicate that either notochord or endoderm acts as a cerebral inductor. The development of the adhesive papillae has been discussed by Cohen and Berrill (1936). They have found three papillae in a lateral half larva of *Ascidiella aspersa* and interpret the origin of such supernumerary papillae as a result of the mosaic pattern. If this interpretation is correct, it might be expected that in larvae with displaced organs, papillae would be found in a variety of abnormal locations. On the contrary, papillae in abnormal larvae are always associated with the endoderm. This fact furnishes further evidence in favor of the suggestion of Tung that papillae appear to be evoked in the ectoderm by the underlying endoderm.

From the foregoing discussion we come to the conclusion that the organization of the unfertilized egg of the ascidian is similar to that of the fertilized egg. In both there exist different kinds of oöplasmic substances from which different tissues develop. These substances, however, are not strictly mosaic; they still possess a certain capacity for regulation. Some organs, such as papillae and sensory cells, seem incapable of self-differentiation. Their development might be dependent upon extrinsic factors.

Summary and Conclusions

1. The unfertilized eggs of *Ciona intestinalis* after centrifuging can be cross-fertilized. The majority undergo cleavage but rarely develop normally.

2. The first cleavage furrow may lie in any plane relative to the position of polar bodies or the axis of centrifuging.

3. In larvae derived from strongly centrifuged eggs, tissues and organs were often displaced from their normal positions.

4. Endoderm and ectoderm appear to be relatively equipotential.

5. There is no evidence that the differentiation of the neural tissue is dependent upon other tissues with which it is in contact.

6. Mitochondria may be displaced from muscle cells and appear in

the neural or ectodermal cells, where they are not transformed into myofibrillae.

7. The number of papillae developed by the abnormal larvae varied from none to 3. They are always adjacent to the endoderm which it is suggested may evoke their formation in the ectoderm.

8. The number of sensory pigment spots in the abnormal larvae varies from none to 4. They are always associated with notochordal cells.

9. The organization of unfertilized eggs is found to be strikingly similar to that of fertilized eggs in respect to oöplasmic substances and the capacity of regulation.

10. The elongation of the notochord is not always accompanied by the elongation of the neural plate or tube.

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THE DEVELOPMENT OF THE BUD IN BOTRYLLUS

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In the following account an attempt is made to describe the development of the bud in *Botryllus* in a manner directly comparable with the development of an egg, in an effort to bring out certain essential simplicities in this direct type of development. The subject itself is not new, but it is believed that the treatment is. Of the older papers those of Hjort (1896) and Pizon (1893) are outstanding. Hjort's accounts were concerned with the significance of the primary germ layers, or rather their lack of significance, in the asexual development of *Botryllus*. Pizon, on the other hand, described primarily the formation of young colonies rather than bud development as such. Also his work was marred by a completely erroneous account of the origin of the gonads, gonads being considered to arise in the oozoöid, in conformity with the opinion of Weismann, and to be transported to the developing buds of succeeding generations until sexual maturity was finally attained. This was a false conception, and the description given here is very different.

Origin and Nature of Bud

The bud of *Botryllus* first appears as a small disc-like thickening of the atrial epithelium on each side of the body, immediately anterior to the gonads. A bud appears on each side, while just posterior to them hermaphrodite gonads continue to develop. The atrial epithelium is of ectodermal origin, and apart from its involvement in the process of budding plays its part apparently only as a limiting membrane. There is, in other words, no reason to suppose that the cells of the atrial epithelium have become in any way specialized. Their formation as an epithelium is a matter of tissue organization and implies nothing in regard to cell specialization.

The disc of atrial epithelium concerned in budding is overlain externally by a similar area of epidermis. This tissue is likewise an epithelium and of ectodermal origin, but its association with test or tunicin production suggests that it has special chemical activities in addition to serving as a limiting membrane. That is, its constituent cells have probably acquired a certain degree of individual specialization.

The double disc of cells forming the initial bud is shown in Fig. 1, in optical section. In Fig. 1, *A* it is shown in relation to its subsequent developmental cycle inasmuch as three stages are shown while yet in organic continuity. In the largest and oldest of the three generations, viewed from the ventro-posterior side, the vascular connection with the circulatory system of the colony is clearly seen. The zoöid is fully active and the contained eggs have developed as far as the gastrula stage. The zoöid bears on its right side a bud about one-fifth its own length in which the organization is virtually completely expressed but is far from being functional. This bud has also formed vascular connection with the colonial system. In turn it bears a bud in the first or disc stage. Two features may be emphasized. A high degree of structural organization is attained at a relatively small size, and the size of the bud in the disc stage is minute when compared both with its size at the end of development and also with that of the developing egg. In Fig. 1, *B* the bud disc is shown, on a larger scale, in relation to the adjacent structures of the parent bud.

Polarity

The question of origin of polarity in ascidian buds has been discussed before (Berrill, 1935, 1936). In every case where organic continuity is maintained between bud and parent, and *Botryllus* is no exception, the polarity of the bud is clearly a derivative of that of the parent. Both the antero-posterior axis and the left-right axis coincide with those of the parent zoöid and must exist from the beginning.

Development of the Bud as a Whole

The simplest conception of the developing bud is that of a mass of tissue expanding during a certain period. This is shown pictorially in Fig. 2. The larger drawing in this figure represents accurately the linear growth plotted against time. The various cross-sections within the cone of growth represent certain developmental stages of special interest. At 26° C. the time units are days, and at this temperature development is completed on about the fifteenth day. If the value of the time unit is coördinated with the temperature coefficient, the cone becomes a constant expression of growth for all temperatures. The growth curve is the usual sigmoid characteristic of developing organisms in general.

At a given temperature, development to the complete functional stage has a specific duration. Equally striking is the subsequent history of the individual so formed. If the temperature is 26° C. and the developmental period fifteen days, the individual lives, feeds, and grows a

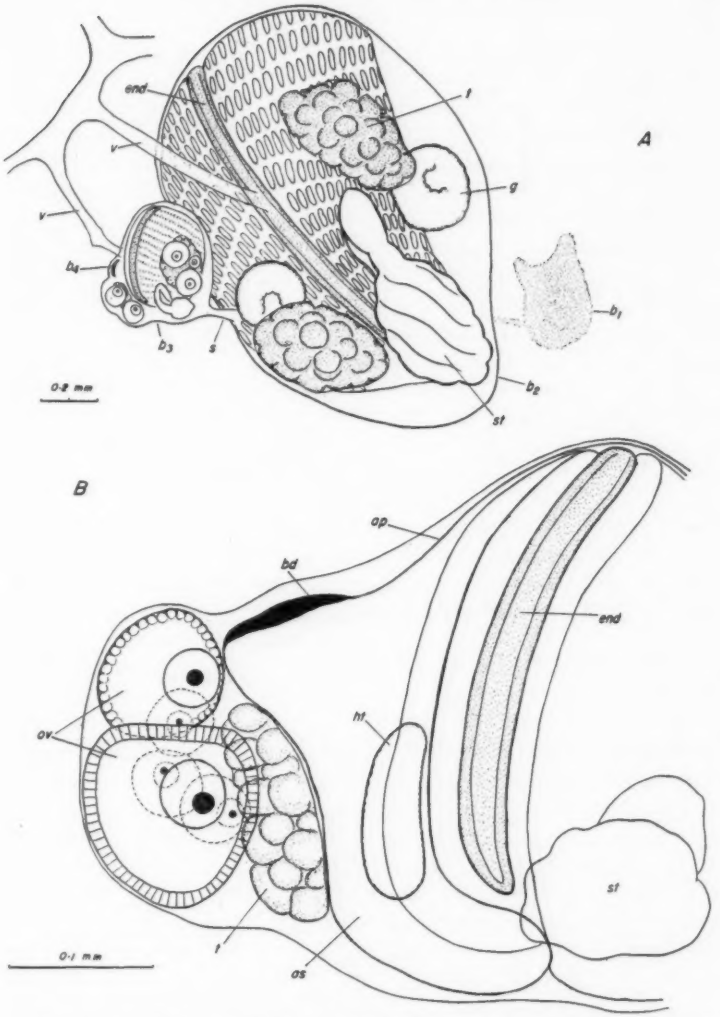

FIG. 1, *A*. Four generations representing complete cycle of zoöid. b_1, "ghost" of autolysed zoöid of preceding bud generation; b_2, ventral view of active zoöid bearing right bud only and containing developing eggs in gastrula stage. b_3, bud borne by active zoöid, with rows of definitive stigmata about to become perforate, and in turn bearing bud of next generation in its initial disc stage (b_4). Both the active zoöid (b_2) and its bud (b_3) are connected with the colonial circulatory system by their ventral ampullary vessel, *v*.

B. Part of b_3 at a higher magnification, showing general relationship of bud disc, atrial epithelium, and gonads. This stage is drawn at the same magnification as those of Fig. 4 and follows as a stage of bud development Fig. 4, *F*.

as, atrial sac; *ap*, atrial epithelium; *bd*, bud disc; *end*, endostyle; *g*, gastrula; *ht*, heart; *ov*, ovum; *s*, bud stalk; *st*, stomach; *t*, testis; *v*, ampullary connecting vessel.

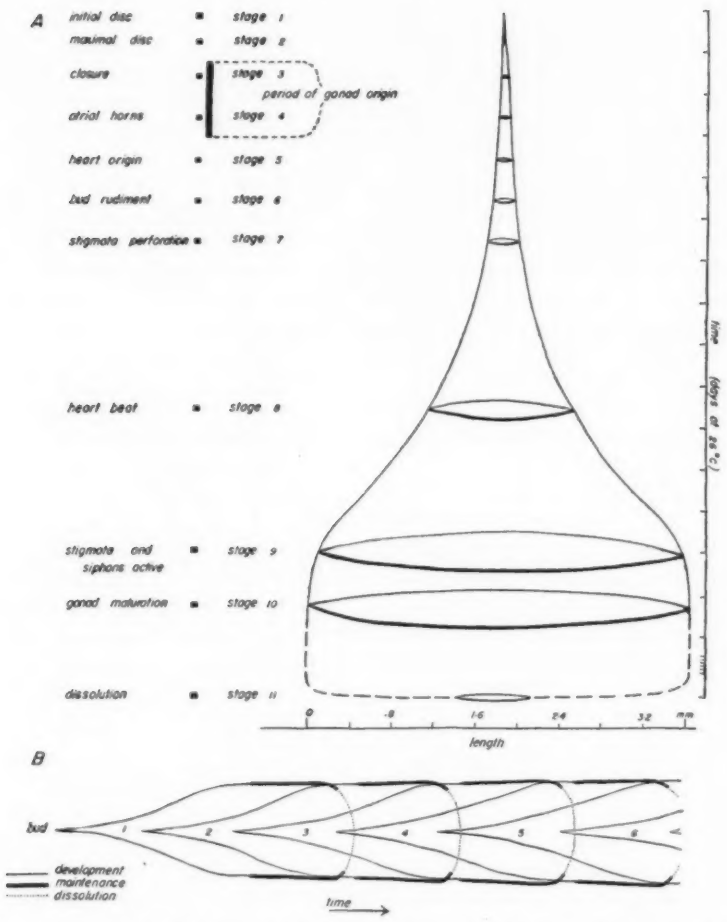

FIG. 2, *A*. Critical stages of bud development in relation to growth curve and whole life cycle of zoöid. The time scale is abbreviated for the maintenance or active period.

B. Similar growth curves for five successive generations indicating overlapping of generations and the three phases of development, maintenance and dissolution.

little, for about seven days. After this a period of dissolution and autolysis occupies a further two days. In Fig. 2, *A*, these last two phases are indicated against a condensed time scale. Their duration is

related to temperature in the same way as is the first or developmental phase.

As shown in Fig. 1, *A*, the first or disc stage in bud development appears when the parent zoöid is itself but one-fifth grown and in turn still attached to its parent. In other words, there is a great extent of overlap in the life cycles of successive generations. This is shown in Fig. 2, *B*. Five complete cycles are shown in the form of overlapping growth cones related to one time scale. It can be seen that in any temporal cross-section three generations in their respective existence phases will occur. It may also be seen that the final phase of dissolution and autolysis commences immediately after the bud of the succeeding generation reaches its full size and ends at about the time the bud attains its maturity. For each generation the developmental phase is shown in light line, the mature phase in heavy line, and the dissolution phase in dotted line.

Early Development of the Bud

Throughout development the epidermis, arising in continuity with the epidermis of the parent, plays almost no part other than to form more epidermis conforming in area and shape to that of the organism arising from the atrial tissue. For the most part therefore it will be ignored.

The youngest stage so far detected consisted of eight atrial cells forming a disc of three cells, more flat than cubical, in cross-section. The whole disc was about thirty micra in diameter, and is shown in surface view in Fig. 3, *B*, and in optical section in continuity with the squamous atrial epithelium in Fig. 3, *C*. Figure 3, *A* represents an arc of the egg of *Botryllus* drawn to the same scale to show the relative size of the egg and the bud rudiment.

As the number of cells constituting the disc increases, the area of the disc increases and the constituent cells change from a sub-cubical to a columnar shape. This leads one to suspect that the columnar condition is typical but in the earliest stage the transition to the surrounding squamous atrial epithelium is so short that the columnar condition can be only partially expressed.

In Fig. 3, *D* the disc stage is shown at its maximum size. With further increase in area, or cell number, it curves into an arc, into a hemisphere, and eventually into a closed sphere (Fig. 3, *E, F, G, H*). The sphere becomes pinched off from the atrial epithelium from which it originated, and the bud remains connected with the parent primarily by an epidermal stalk. Two phases may accordingly be distinguished, an expanding disc phase, and the phase of continuing expansion during

which the disc curves into a hollow sphere. A further feature of considerable significance is associated with the second of these phases. This is shown in Fig. 3, *G* and *H*, in which the gonads are already appearing. In the closed sphere stage shown in Fig. 3, *H*, the sphere proper is shown in optical section. In addition certain cells stand out clearly in surface view. These consist of three primary ova and a number of small more ventrally placed cells destined to form the testis. In the younger stage in Fig. 3, *G*, even before the sphere has closed, four cells can be seen which, from their position and shape, are undoubtedly four primary ova. These and later reproductive cells arise by extrusion or delamination from the wall of the sphere. The gonads arise therefore in a remarkably precocious manner.

In Fig. 3, *H*, the epidermis shows definite evidence of active morphogenesis, for the distal evagination is the rudiment of the epidermal stolon that unites eventually with the colonial circulatory system. The disc, hemisphere, and closed sphere stages are shown on one-half the scale of Fig. 3 in Fig. 4. In this are also shown three subsequent stages. Apart from the growth and elaboration of the gonads, which is described in detail later in this paper, these six stages represent stages in a single continuous process. The process of expansion and folding that changes a disc into a hollow sphere continues so that the sphere becomes concave along several facets or arcs. As the anterior arc continues to expand, two vertical folds appear. At the same time an evagination appears in the posterior arc. These are shown in Fig. 4, *E*. The anterior folds gradually extend posteriorly until they divide the single vesicle into a median and two lateral chambers. These three units are the central pharyngeal chamber and the pair of lateral atrial chambers (Fig. 4, *F*). At the same time the posterior evagination grows out to form the rudimentary stomach and intestine. When the primary subdivision into three chambers is complete, two small evaginations develop from the central chamber. Median and anteriorly a small bulge becomes the neural mass, while a somewhat larger evagination from the left posterior wall represents the developing heart. Therefore, apart from the segregation of lateral masses from the wall of the sphere to form the gonads, all the principal divisions of the *Botryllus* zoöid are produced, pharyngeal and atrial chambers, intestine, heart and neural complex by a simple process of progressive folding of an expanding sheet of tissue. These are all clearly shown in Fig. 4, *F*.

Later development is primarily an elaboration of detail of each of these divisions. Figure 1, *B* is of the same scale as Fig. 4 and demonstrates both the extent of growth and elaboration that occur by the time the developing atrial epithelium in turn has formed its bud disc. The

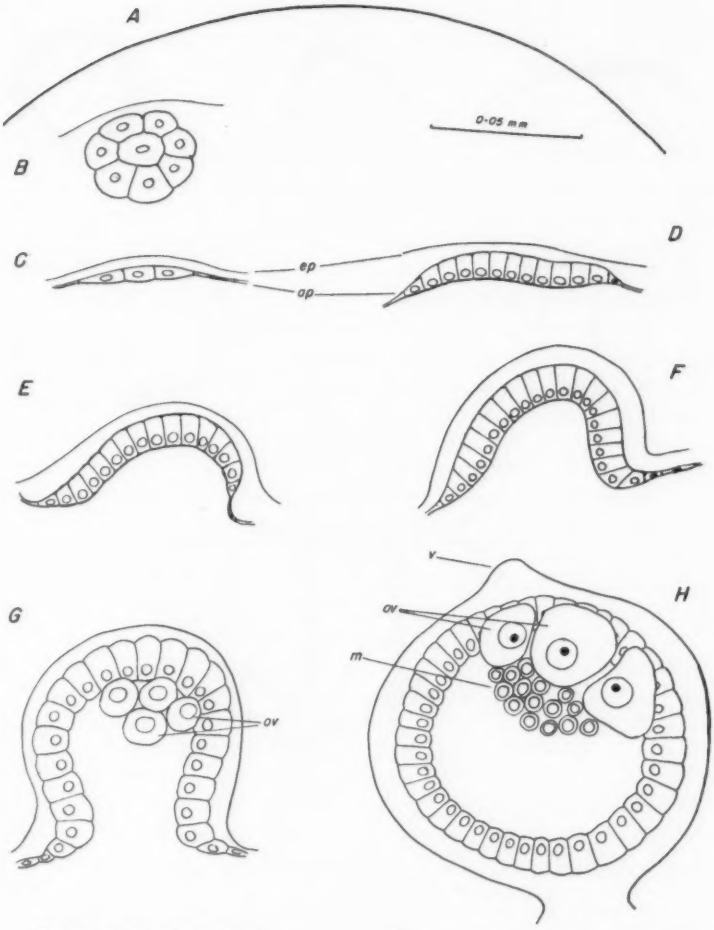

FIG. 3. Formation of bud.

A. Part of circumference of mature egg drawn to same scale for comparison of size.

B, C. Surface view and optical section of initial disc (stage 1).

D. Optical section of maximal disc (stage 2).

E, F. Arching of disc to form sphere or vesicle.

G. Bud vesicle beginning to close proximally, and showing lateral segregation of four cells destined to become mature ova, extruded into space between inner and epidermal vesicles.

H. Later stage (stage 3) with vesicle closed, epidermal ampullary vessel protruding distally, three presumptive mature ova and a number of male cells all extruded from the lateral wall of the vesicle, to lie outside it. A similar condition exists on the opposite side of the vesicle, not shown in the figure.

ep, epidermis; *ap*, atrial epithelium.

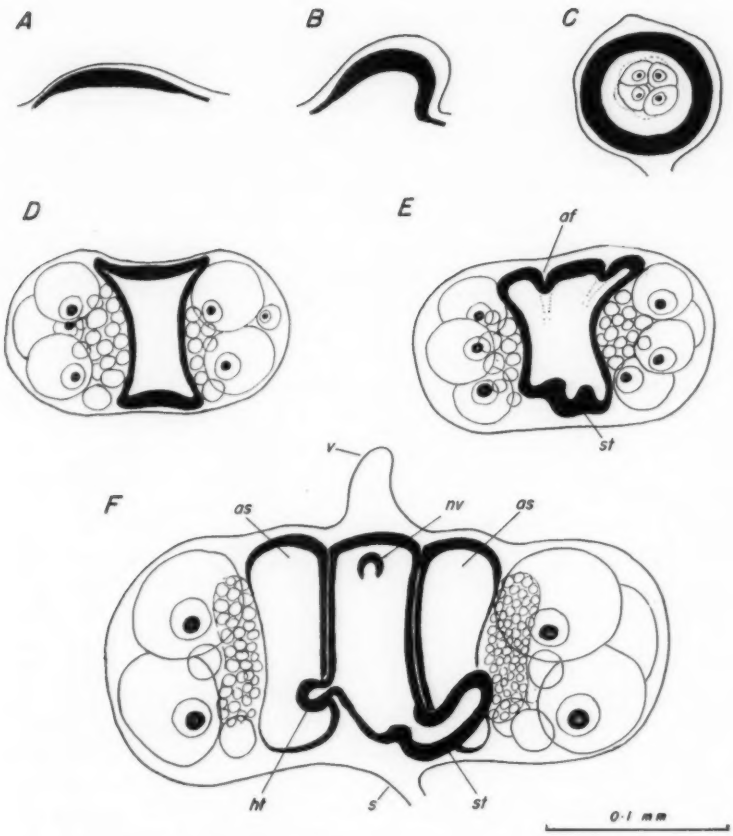

FIG. 4. Early development of bud.

A. Maximal disc (stage 2), from left side.

B. Hemisphere stage, from right side.

C. Closed vesicle (stage 3), from right side, with gonads extruded from wall.

D. Continued gonad segregation, stage viewed from ventral aspect.

E. Formation of atrial folds and intestinal outgrowth (stage 4), from ventral side.

F. (Stage 5.) Origin of heart, intestine and neural vesicle, and completion of subdivision of vesicle into central pharyngeal chamber and lateral atrial sacs, from ventral side. The succeeding stage (stage 6) is shown on the same scale as Fig. 1, *B*.

af, atrial fold; *as*, atrial sac; *ht*, heart; *nv*, neural vesicle; *s*, stalk; *st*, stomach; *v*, epidermal ampullary vessel.

bud disc appears at a precisely definable stage in the whole development and is to be regarded as an essential and definite constituent part of the organization of a specific stage.

Later Development

Development of Stigmata.—Gill slits develop as perforations of the combined pharyngo-atrial wall. This is the case both for the organism developing directly from the egg and for the developing bud. In the first case atrial sacs grow in on each side of the embryo and come into contact with the pharynx wall. Gill slits appear only within the area of contact. In the bud the equivalent double wall is formed, as already described, by the downgrowth of the pair of anterior folds that divide the primary vesicle into the central and lateral chambers, as shown in Fig. 4, *E* and *F*. The two walls are shown in Fig. 5, *A* at a stage intermediate between the preceding two. Only when this double wall expands to about ten times its linear size does stigmata formation become evident. The first indication is the appearance of an alternating thickening and thinning (spatially) of each of the two component epithelia separately, as shown in Fig. 5, *B*. The thick ridges run dorso-ventrally from the mid-dorsal line to the endostyle and each represents a row of stigmata. Between the ridges the epithelia flatten out as the interstigmal tissue. This condition is definitely associated with the stage bearing the bud disc stage of the next generation.

As the two layers of ridges or thickenings increase somewhat in depth they come into contact and fuse at a series of points along each pair of ridges. Perforation occurs at these points to form the rows of stigmata in their first definitive stage. The first perforate stage is shown in Fig. 5, *C* and *D*. Subsequent development consists of an elaboration of each of the units thus formed. No more will be added. Perforation of the fusing wall occurs at a definite and precisely definable stage of development. At this same stage other features of the developing pattern will be at a constant associated condition.

The stage of development of the bud of the next generation conforms to this relationship just as any other feature, and is near the hemisphere stage of vesicle formation (Fig. 3, *F* and *G*). In other words, the bud itself is an integral part of the whole organization pattern and the time and place of its inception are as sharply defined as that of any other unit structure in the developing organism.

At perforation each stigma in surface view consists of a rosette of about six cells (Fig. 5, *D*). Each constituent rosette of a row continues its development as a unit. With multiplication of its seemingly unspecialized cells the central aperture bordered by the cells expands and

elongates, so that the size and length of each stigma increase progressively. When the cell-multiplication is almost terminated each cell develops short cilia. Further growth of each stigma to approximately

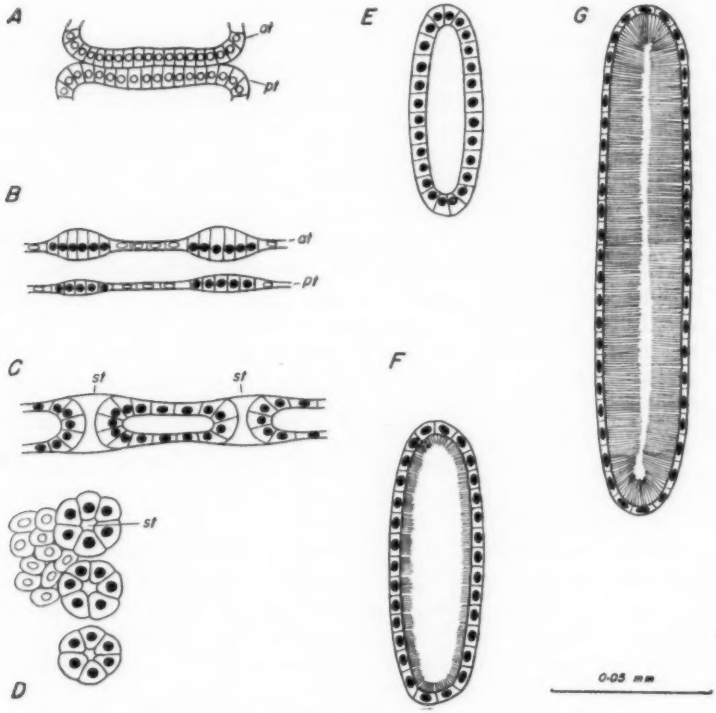

FIG. 5. Development of gill slits (stigmata).

A. Double layer formed of inner atrial wall and of pharyngeal wall.

B. Alternate thickening of atrial wall and corresponding thickenings of pharyngeal wall, each such paired thickening representing cross-section through ridge destined to become row of stigmata.

C. Equivalent section to *B*, but of perforate stage.

D. Surface view of *C*, showing three stigmata rosettes in initial perforate condition.

E, *F*, and *G*. Three stages in subsequent growth and differentiation of a single rosette to form a functional gill slit.

at, atrial wall; *pt*, pharyngeal wall; *st*, perforate stigmata.

double its size takes place as the result of change in cell size and shape, and at the same time the cilia grow until they almost touch those from the opposite side (Fig. 5, *F* and *G*).

Accordingly the following features are evident in the development of the gill slits: There is a primary condition in which atrial and pharyngeal epithelia are present and in virtual contact. The basic pattern is expressed as a series of ridges, each with a series of swellings in each tissue some time before stigmata formation. Perforate stigmata appear at the points of fusion between the two tissues, while subsequent growth consists first of a period of cell multiplication and then of a terminal phase of individual cell expansion and cyto-differentiation.

Development of the Gonads.—The origin of the gonads has been described already. Their subsequent development as a unit organ is, however, of some interest, as is that of a single ovum. Figure 6 shows sections through a number of stages. Figure 6, *A* represents a section of a stage immediately following that seen as a whole mount in Fig. 3, *H*. The originally thick wall of the internal vesicle is divided into the thin atrial wall and the massive developing gonad. The gonad here consists of two primary ova and a mass of loose cells representing a few rudimentary ova and many male cells. Cells are added to the collection over a considerable period from some parts of the inner retaining wall. In other words, as the lateral walls continue to grow, the splitting into inner atrial and outer gonadial components continues in marginal regions previously incapable of such splitting by virtue of insufficient cells. This is shown in Fig. 6, *B*, a section passing transversely across the anterior end of a bud at a considerably later stage, a stage intermediate between those shown in Fig. 4, *E* and *F*.

The section shown in Fig. 6, *C* illustrates several points of interest. The inner atrial epithelium is entirely distinct in kind and in space from any part of the gonad. The form of the lobular testis becomes apparent in spite of the small number of its constituent cells. And in the case of both ovary and testis there is a residual mass of cells unincorporated into those organs. In the case of the ovary, the small inner ova never grow and mature. The residual cells of the testis may or may not develop into testicular lobes, depending on the degree of belatedness of their segregation. Virtually the complete form of the testis is to be seen in the stage represented in Fig. 6, *E*, even though the testis here is less than one-quarter its final size (in linear dimension). The form is almost fully expressed, but its histo-differentiation is indiscernible. In fact the final differentiation into condensed and tailled spermatozoa occurs only after the full size of the developing bud is at last attained.

In the ovary those ova segregated from the vesicle wall in the first phase of gonad formation (in number from one to four) grow and mature. Those formed later remain close to the size at which they were segregated. The primary ova, as far as can be determined, in-

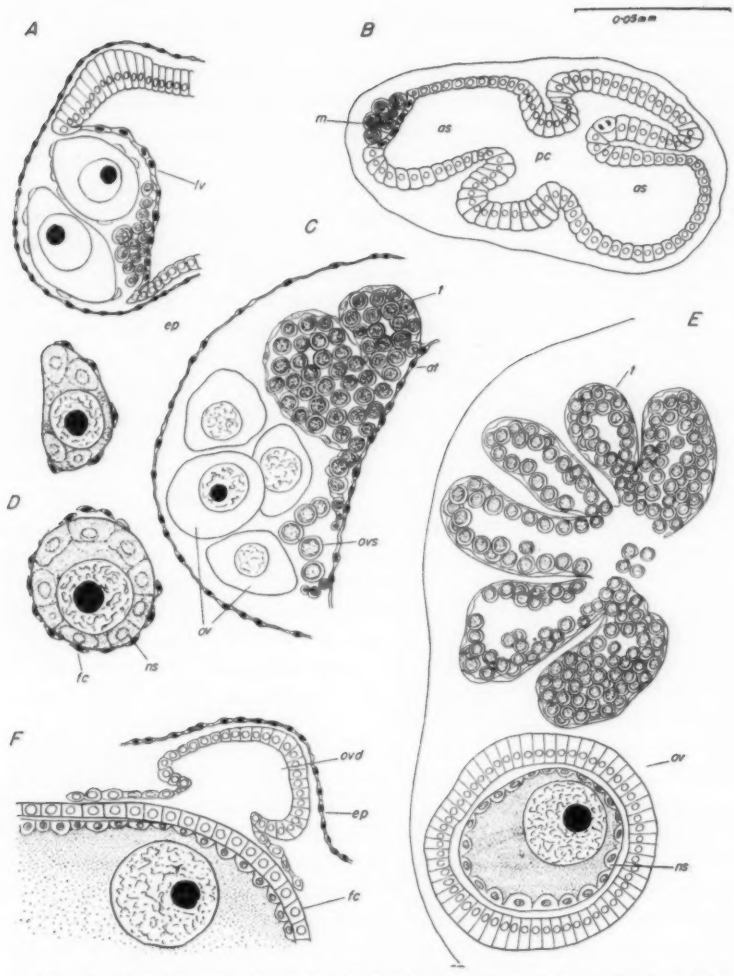

FIG. 6. Development of gonad. All drawings from actual, not merely optical, sections.

A. Left half of advanced vesicle (between stages 2 and 3) showing differentiation of lateral wall into atrial epithelium, male cells, and presumptive mature ova.

B. Frontal section of later stage (between stages 4 and 5) in anterior region, showing continued segregation on left side of male cells from the lateral wall.

C. Left side of bud at stage 5 showing four presumptive mature ova, a few undeveloping ova, and precocious lobulation of testis.

D. Two isolated ova with follicle and nurse cells.

E. Gonad of stage 6, showing lobular testis, and a single ovum with numerous follicle and nurse cells.

F. Part of ovum at its maximum size, showing nurse cells, follicle cells, and developing oviduct of same origin as follicle cells.

at, atrial epithelium; *ep*, epidermis; *fc*, follicle cell; *lv*, left wall of primary vesicle; *m*, male cells; *ns*, nurse cells; *ov*, ovum; *ovd*, oviduct; *ovs*, secondary ova; *t*, testis.

clude other cells from the first. No stage, with the possible exception of those shown in Fig. 3, has been seen in which the ova proper are without accessory cells. These cells are of two kinds, a few flattened follicle cells clinging to the surface, and an equally small number of nurse cells completely within the cytoplasm of the ovum. Following multiplication, the outer surface cells become columnar, as in Fig. 6, *D*, and eventually flatten again as they give rise to the egg chorion. The nurse cells also multiply and are clearly involved in the growth of the ovum. They are eventually extruded into the perivitelline space as the

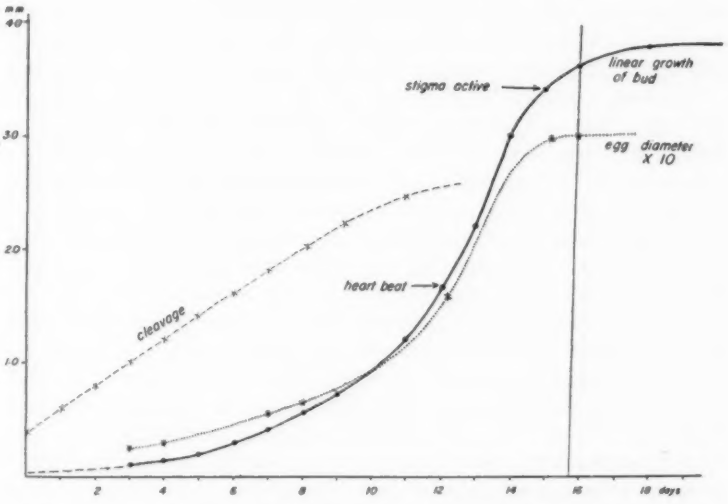

FIG. 7. Growth curves for linear growth of bud, and of single egg × 10. On the same chart is also shown a cleavage curve indicating the geometrical increase in cell number associated with growth.

inner follicle cells or "test" cells of the mature ovum (cp. Berrill, 1929).

Thus the development of the gonad as a whole is a comparatively complex process. Yet the ova and spermatozoa attain actual functional maturity at virtually the same time and almost at once after the bud as a whole has become active. Either some factors external to the gonads suddenly terminate growth and multiplication and enforce final differentiation to coincide with that of the rest of the bud, which is unlikely, or the development has from the first been approaching a condition of equilibrium permitting final cyto-differentiation. That this last is the

case is shown by a comparison of the growth curves for the bud as a whole and of a single ovum. The curves for the linear growth of the bud and the ovum, when reduced to equivalent scales, are practically identical, as may be seen in Fig. 7. Both are sigmoid curves indicative of an approach to and attainment of a " steady state."

Cell Division and Differentiation.—In all tissues of which the cells finally exhibit a marked degree of structural specialization or differentiation, the structural details become visible only at or toward the close of the phase of cell multiplication. The time at which this occurs varies greatly. In the case of spermatozoa, condensation and elongation occur only after the rest of the organism has as a whole become functionally active. In the case of ova, as distinct from their associated follicle cells, growth and differentiation without division occupy almost the whole developmental period. Muscle cells of the heart and body wall stop dividing and become greatly elongate when linear growth of the whole organism is little more than half complete. Cessation of division and subsequent formation of long cilia in stigma cells occurs very late, but is complete before elongation of spermatozoa commences. In the case of ova and muscle cells, at least, there is very considerable growth after cell division has come to an end. Growth of the organism as a whole accordingly conforms to a typical curve in spite of the fact that the growth is in part due to cell multiplication and in part to cell growth without division. The growth curve for a single ovum is similar to that of the whole organism. It seems clear that the developing bud grows at a rate characteristic of an approach to a " steady state," and that the growth of the parts, whether based on cell division or not, is not a group of independent processes coöperating to form the whole, but must be governed by the whole. Cell division as such becomes, in this view, a condition and tool of the whole developmental process rather than in any sense a basic cause. Otherwise the coördination of the varying times at which different cell types cease division and differentiate becomes virtually unaccountable. In a comparison of the growth of a non-dividing ovum with the growth of a group of cells by multiplication, it appears that the rate of volume-increase is quite independent of division processes.

Summary and Conclusions

The bud arises as a disc-like thickening of the anterior atrial wall, consisting of a small number of columnar cells transformed from the atrial epithelium, overlain by an equivalent area of unmodified epidermis. The polarity of the disc and subsequent organism is an extension of that

of the parental tissue, with regard both to the antero-posterior and lateral axes. Development itself is fundamentally extremely simple and direct. After the completion of development there is a phase of functional activity and a phase of autolysis and dissolution. For any given time-temperature scale the duration of these last two phases is as specific and determined as that of the developmental phase. Of the two tissues constituting the bud disc the epidermis forms only more epidermis, though acquiring the form of a whole organism including the ventral stolonic outgrowth. The atrial component of the disc forms everything else. As the disc expands, by means of cell multiplication, it transforms progressively into a hemisphere and eventually into a hollow sphere attached by a narrow stalk to the parental tissue. Two folds develop anteriorly and divide the vesicle into two lateral and one median chamber. The lateral divisions represent the atrial chambers, the median the pharyngeal sac and from it three evaginations are formed representing the heart, neural mass, and intestine respectively. Later development is primarily an elaboration of these unit-regions. As an example, the formation, growth and differentiation of gill slits in the pharyngeal wall is described in detail. The essential pattern of the stigmata is apparent even before they become perforate. Each stage in their development is precisely correlated with specific stages in the development of the whole organism.

The bud anlagen of the succeeding generation appear as discs in the anterior wall of the left and right atrial chambers at a specific stage in the development. This stage is that in which rows of stigmata, while not yet perforate, are represented by ridges or folds of the pharyngeal wall. At the time of perforation, the buds are approximately at the closed vesicle stage. The buds, in fact, are to be regarded as essential constituents of the organization pattern, appearing and developing in time and place in a manner strictly analogous to that of any other unit structure.

The gonads segregate as a mass from the lateral walls of the bud at an extremely precocious period, even while the primary vesicle is in process of formation. Once segregated, they in turn develop as a seemingly independent unit structure. The testes show the final lobular form virtually as soon as sufficient cells are present for its expression. Ova, apart from the associated internal and external follicle cells, grow and differentiate without dividing. They mature finally at the same time as the spermatozoa which cease dividing and differentiate later than any other tissue of the bud.

The development of each tissue is fundamentally the same. A period of cell multiplication is followed by a phase of final differentiation. This

last phase may or may not include a period of cell enlargement, depending on the cell type to be formed. In the case of ova the multiplication phase is barely present at all and the second phase occupies most of the developmental period, involving enormous growth. In spermatozoa the case is reversed and the final phase is extremely brief and actually involves reduction in cell size. Muscle tissue lies between these two extremes, while most other tissues approach more the condition of spermatozoa.

The whole development of the bud and that of its component parts is therefore as direct a process as can be conceived, without there being any indication of the divergence to form tadpole larvae associated with egg development. Cell multiplication continues to a greatly varying extent in different parts and tissues, while the linear growth of the whole or of a non-dividing ovum follows a regular sigmoid curve typical of an approach to and attainment of a "steady state." In fact, the development of the bud is essentially such a unitary process that "wholeness" can be said to be the most outstanding feature of the organism not only in its final functional phases but of every moment of its existence, and especially of the beginning. It is virtually as though organization is present from the first, though the extent of its visible expression is closely correlated with and limited by quantity of available material at every moment of development.

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SIZE AND MORPHOGENESIS IN THE BUD OF BOTRYLLUS

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The bud of *Botryllus* first appears as a disc arising in the anterior wall of each of the atrial chambers. The disc grows a little and then transforms into a sphere. The size to which the disc grows before it starts to transform varies among different bud generations in a *Botryllus* colony, increasing slightly with each successive generation of buds. The present account is primarily a comparison of the development of the buds arising from the smaller discs of early generations with the large discs of late generations, within the colony formed from a single fertilized egg. In addition to differences in size of bud primordia from early and late generations in the colony, there are usually size differences between the bud primordia of the right and left sides, that of the right side being the larger.

The development of a bud has as its basis a continuous material expansion from the small group of cells constituting the primary disc to a functional bud of several thousand times its volume and cell number. The significance of this expansion is paramount. As the disc expands in area it becomes curved into a hollow sphere. As the sphere expands, its surface folds inwards to divide it into three chambers, the major territories of the body. With continued expansion, further surface folding occurs to divide off smaller territories such as neural mass, heart, and intestine. It can be said that for each successive size the material (mass, area, cell number, etc., however it may be expressed) present at that moment expresses virtually every character of the final organization that is not inhibited by the limitation of size itself.

Each bud disc arises from the atrial epithelium as a group of cells that gradually acquire a columnar form. The epidermis forms an equivalent overlying component of the disc, but plays a relatively minor part in the subsequent development. Since every disc of atrial cells has to develop from the general atrial epithelium, there is almost certainly no real minimum size that can be compared in different generations. On the other hand, the disc in every case grows to a certain extent before changes in form begin, and the size or cell number of the disc at its maximum size, which is a precise stage, is a value readily compared.

FIG. 1. Formation of bud vesicles, all in optical section. *A*, *F*, and *I* are three maximal bud discs. *A–E* represents vesicle formation from small right maximal disc from zoöid of young colony. *F–H*, the smaller left maximal disc of same series. *I–M*, vesicle formation from large right maximal disc from zoöid of a mature colony. *M* and *N*, right and left vesicles from same individual and forming three and one mature ova respectively.

The smallest maximal disc commonly seen consists of about six cells in optical section, the largest of about fourteen cells, or a difference of about eight times in volume of tissue or number of cells present at this stage. Figures 1, 2, and 3 represent a comparison of the development of two sizes of maximal right bud discs, from early and late generations respectively. In Fig. 1, *G*, *H*, and *N*, the smaller left buds are also shown. In this figure several features of comparative interest are clear. The relative difference in size of three maximal disc stages is maintained in the subsequent stages of hemisphere and sphere. In optical section these maximal discs have 5, 8, and 14 cells respectively (Fig. 1, *F*, *A* and *I*), representing totals of about 21, 48, and 150 cells (ratios 1 : 2·1 : 7·1). In the corresponding closed sphere or vesicle stages, optical sections show 9, 15, and 25 cells respectively (Fig. 1, *H*, *E* and *M*), representing cell totals of about 33, 75, and 210 (ratios 1 : 2·2 : 6·3). From these values two facts emerge. The ratio of cell numbers representing the smallest and largest maximal disc illustrated is about 1 : 7. The same ratio holds for the closed vesicle stage, and it is evident that whatever the size of the maximal discs, the transformation is correlated with an increase in cell number of about one and one-half times that of the disc.

Morphogenesis is thus independent of absolute cell number, but closely dependent on relative cell number.

In Fig. 1 two other features are evident. The relation of morphogenesis to cell number is the same in the epidermis as in the atrial tissue. The epidermis conforms in size and shape to the inner tissue, and as may be seen in Fig. 1, *E* and *K*, the protrusion foreshadowing the ventral ampullary vessel appears in both small and large vesicle stages, and in spite of the very early stage in development as a whole.

It is of greater interest that gonad tissue is segregated from the right and left lateral wall of the vesicle stage in the large forms but not in the small. These two correlated variations in the vesicle stage, namely degree of segregation of gonad tissue and the absolute size, produce an increasingly marked effect on later stages of development. Figure 2 shows immediately succeeding stages drawn to the same scale as those in Fig. 1. Figure 3 shows still later stages at necessarily reduced scales. In each case equivalent stages are shown for the development of both large and small primordia.

The two primary differences, in size and gonad development, are maintained in an increasingly obvious form. Thus in Fig. 2, the three stages *A*, *B*, and *C* are morphologically equivalent to the stages *D*, *E*, and *F*. In *A* and *D* the folding of the vesicle wall to delimit the primary divisions of the body are just beginning. In *B* and *E* they are com-

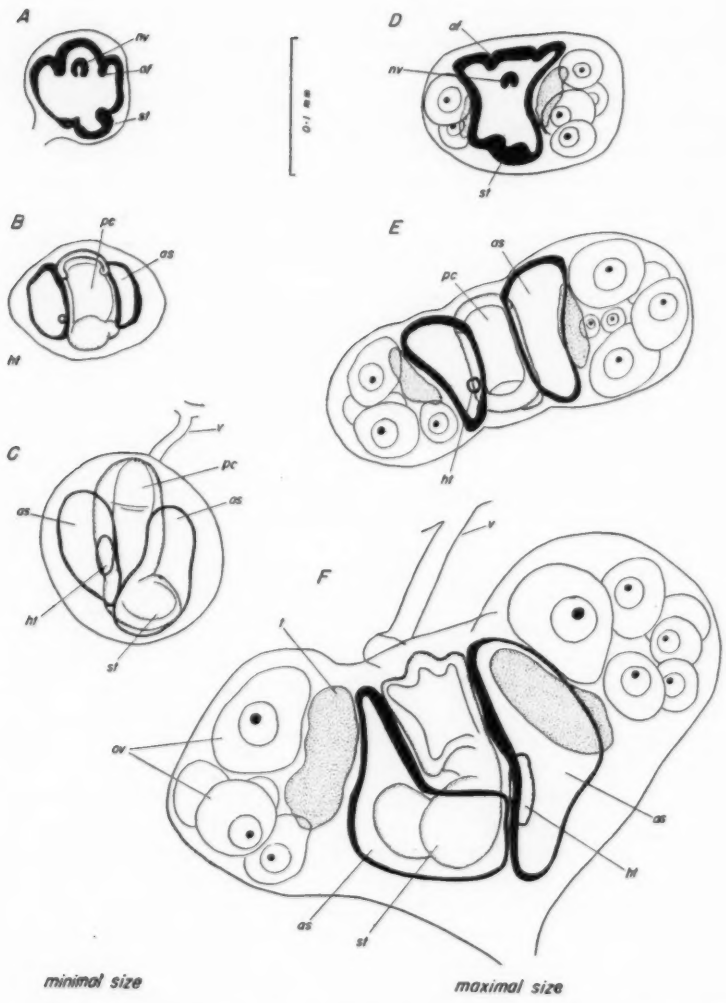

FIG. 2. Development, at same magnification, of series *A–E* and *I–M* of Fig. 1. *A–C*, development of small vesicle, *D–F* of large vesicle, showing differences in size of equivalent stages and in presence and absence of gonads.

af, atrial folds; *as*, atrial sac; *ht*, heart; *nv*, neural vesicle; *ov*, ova; *pc*, pharyngeal cavity; *st*, stomach; *t*, testis; *v*, ventral ampullary vessel.

pleted, and atrial chambers, pharynx, intestine, heart, and neural mass already exist as unit regions. The difference in diameter of the closed vesicle stage shown in Fig. 1, *E* and *M*, is fully maintained. In addition there is the striking difference in gonad development. In the series of stages associated with the small primordium none appear, in the larger they continue to develop and become massive organs on each side between the lateral wall of the atrial epithelium and the epidermis. These differences become more and more pronounced, as may be seen in Fig. 3.

Size and General Organization

While the structural consequences of the primary difference in size become progressively more obvious, one feature needs to be emphasized strongly. The great difference in cell number constituting maximal disc stages is maintained at least in the later closed vesicle stage, the increase being about one and one-half times. The difference in cell number is expressed less markedly in disc and vesicle diameters. In the two main series illustrated in Fig. 1, the diameter of the larger series is about one and two-thirds that of equivalent stages of the smaller series. Excluding gonads for the time being, this difference in linear dimension of the two series is maintained closely in the later stages shown in Figs. 2 and 3 up to and including the active functional stage. Not only is the linear size difference maintained throughout development, but it is equally expressed in the number of such multiple structures as stigmata. In the stages in which stigmata are just becoming perforate and in which they are active organs, the number of rows of stigmata is six in the smaller buds, ten or eleven in the larger, while the number of stigmata per row in the smaller is 12 and in the larger 22. The number of stigmata does not change during development. Thus in the two series the number of stigmata formed is proportionate to size, since both linear dimension of the whole, and the number of rows of stigmata and number of stigmata per row, vary as one to about one and two-thirds.

The difference in whole size of equivalent stages, which is expressed numerically in multiple organs such as stigmata, applies equally to organ size. This is the case for the heart, for in the three stages—primary heart vesicle, initial beating, and final—the same relative size differences are maintained.

Since the relative difference in size between the two series is maintained virtually at a constant level for all stages, it follows that each stage represents a certain degree of expansion in terms of a preceding stage, whatever may be the absolute size. A specific degree of expression of the complete organization is correlated with a certain size or

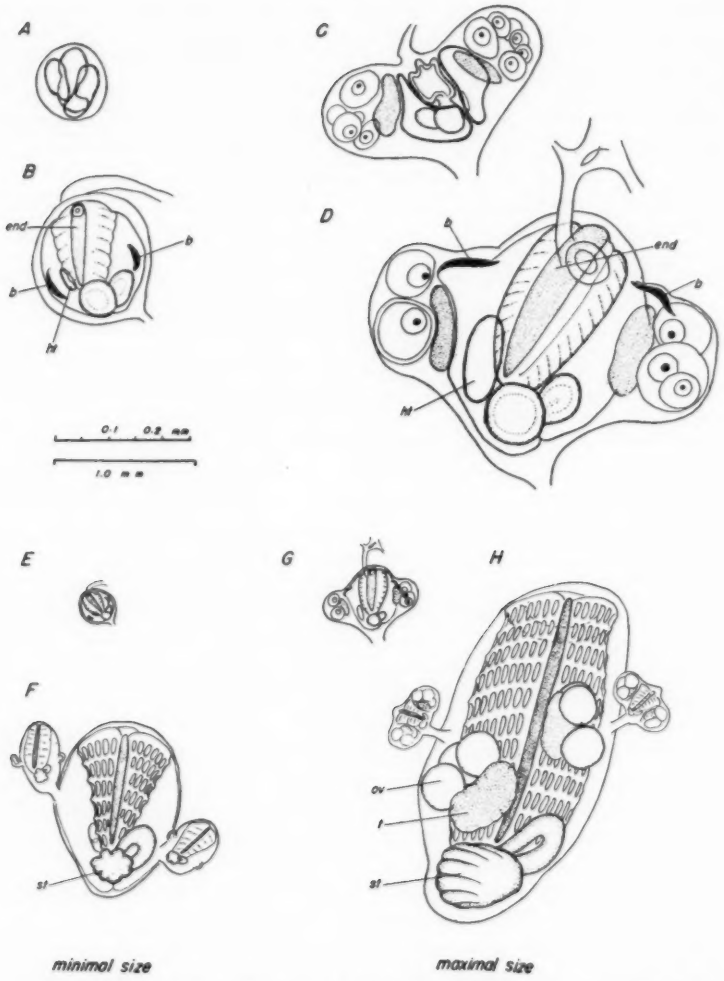

FIG. 3. Continuation of same two developmental series, at two smaller magnifications. *A–B*, *E–F* continued development of smaller bud disc and vesicle shown in Figs. 1 and 2. *C–D*, and *G–H*, continued development of larger vesicle.

For purposes of comparison, *A* and *C* represent at the lower magnification the stages shown in Fig. 2, *C* and *G;* in the same way *E* and *H* are reduced from *B* and *D*. *B* and *D* are equivalent stages and have formed maximal bud discs. *E* and *H* are also equivalent and have become active zoöids. The difference with regard to size, presence of gonads, and length and number of rows of gill slits is obvious.

b, bud disc; *end*, endostyle; *ht*, heart; *ov*, ova; *st*, stomach; *t*, testis.

material quantity. This size is not absolute and is not expressable in actual measurement or cell number, but must be expressed in terms of reference to the absolute size of the maximal disc stage. This is highly significant and will be referred to again.

Development of Gonads and Initial Size

Confining the present account to the two extremes already illustrated, there is a spectacular difference in the condition of the gonads in the two series, one producing mature gonads and the other none. This difference goes back to the first stages of development. In the larger series, the gonads are separated or extruded from the lateral walls of the primary vesicle even before closure is complete. Once separated, the gonads develop apparently as independent unit regions. The separation phase is comparatively brief, and there is no tendency to form gonads except during this precise phase of the whole development. The ova destined to become mature are the first tissue to be separated, testis and prospective immature ova separating a little later.

In the smaller series, no separation of gonads occurs at all during the equivalent phase, and no gonads appear at any later time. Consequently the massive lateral bulges representing the growing gonads in the developing buds of mature colonies are absent altogether in those of very young colonies.

The correlation of presence or absence of gonad separation (and therefore of subsequent development) with the size of the transforming disc stages suggests at once that size itself may be the determiner.

Gonad tissue is separated during a very definite and specific period of bud development, namely, during a period starting before closure of the primary vesicle and lasting until the vesicle is more or less subdivided into its three primary regions. At no other moment in development, either earlier or later, is there any indication of gonad formation. There is no absolute proof that at no other time under any conditions can gonad tissue be separated, but it is reasonably certain that the capacity to produce gonad tissue is definitely limited to the period or phase in which production always occurs. In other words, the gonad primordium is determined and formed at as precise a period in the whole development as is the case for other organs, such as the heart.

Accordingly, if this assumption is made, it is easy to account for the suppression of gonad formation in the development of small buds. The situation is clearest if one compares the largest and smallest of the four vesicle stages shown in Fig. 1. In the largest, three prospective mature ova have separated from the vesicle wall. The extrusion of these par-

ticular cells commences immediately after the attainment of the open hemisphere stage. The gonad material thus separated must represent a certain minimum proportion of the lateral wall from which it arises, of the order of one-quarter to one-half. It is separated when the vesicle cells total about 160 and when there are about 22 to 26 cells in optical section (Fig. 1, *M*). At this stage, in other words, the material from which ova are separated is in the form of a sufficient number of cells for individual cells representing individual ova to be pushed out.

In the equivalent stage of the smallest series, only 7 or 8 cells constitute the optical section, and the region from which gonads and atrial wall should be differentiated consists, on each side, of only 2 cells in optical section. Accordingly the region constituting the prospective ova is at this stage and size inadequately cellular and the separation of ova at this moment becomes mechanically impossible.

Gonad formation, however, is not momentary but occupies a period of time. In the smallest series even the late closed vesicle stage consists of so few cells (Fig. 1, *E*) that at no time during the proper period can gonad tissue be separated. In the largest series the prospective mature ova are extruded before closure, male tissue and prospective immature ova during and shortly after closure of the vesicle, and some additional male cells after completion of the extrusion of female cells.

In series of developing buds of intermediate size all conceivable types of immature gonads should be found. This is the case. In a series slightly larger than the smallest, a sufficient size or cell number is attained before the gonadial phase is completely passed, and some male tissue is separated at the end of that phase. In a somewhat larger series again, and a little sooner, more gonad tissue is separated consisting of prospective immature ova and male tissue sufficient to develop into a lobular testis of submaximal size. Similarly, in a larger but not maximal series, there may be a separation at or before closure of one or two or three prospective mature ova. In other words, a complete grading from none to mature 4-ova gonads exists, correlated both with size of series and with time of separation.

Conclusions and Summary

In successive bud generations within a colony there is a progressive increase in the size of the bud rudiments and zoöids subsequently developing from them. Dealing with material derived from a single fertilized egg, it is possible to determine the relationship or importance of rudiment size to morphogenesis. Development in every case is simple and direct. It consists of the growth of the rudiment to a maximal

disc stage, the conversion of the disc into a sphere, the subdivision of the sphere or vesicle into unit regions, the whole process being accompanied and conditioned by expansion or growth of tissue.

The maximal disc is a precise relative stage. The larger discs, typical of late and mature generations, may be seven times as large in area as those of early generations.

Whatever the size of the maximal disc, succeeding stages bear to it definite growth ratios. A certain percentage expansion or growth of the disc tissue is correlated with a specific developmental stage, whatever the absolute sizes may be. Absolute size must be determined during the initial phase of development before the disc exhibits any tendency to transform into a vesicle.

Ultimate size being thus initially determined, there is variability in the following expressions of size. All organs and regions vary in absolute size, maintaining their proportionate dimensions relative to the whole. Multiple structures, such as stigmata, vary little in absolute size for a given stage but vary in number in proportion to tissue area. The relatively massive gonads, fully formed only in the largest series, are partially or completely inhibited in the smaller.

Gonad formation is essentially a serial separation of the various components during a short phase of development, lasting from the open hemisphere stage to the expanded closed vesicle stage. If the size of the whole permits separation of each component as discrete cells at the proper time for separation, maximal mature gonads will be formed and develop. If size is so reduced that the various components cannot be materially separated as cells, separation is inhibited and no gonads will develop at the normal or any other time. With successive increases in size from this last condition an adequate cellular state is reached, at first including the later phases of the gonadial period and progressively including the earlier, so that a series of immature gonads appear in the inverse order of normal maximal development. Prospective mature ova do not appear at a time normal for the appearance of prospective immature ova or for male cells. Gonad components that do not separate at their normal time do not appear at all.

THE EFFECT OF SALINITY UPON THE RATE OF EXCYSTMENT OF ARTEMIA

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Introduction

The phyllopod crustacean *Artemia* lives and reproduces in natural and artificial brine pools and lakes in many parts of the world. It tolerates an extreme range of salinity, pH, and other environmental conditions, although it is relatively intolerant of certain substances such as potassium [1] (Martin and Wilbur, 1921; Boone and Becking, 1931). *Artemia* does not require brine, or other environmental extremes, since it completes the life cycle in ordinary sea water in the laboratory, but it is defenseless in nature and is quickly destroyed by predators except in environments which exclude them.

Artemia are abundant in evaporating ponds on the margin of San Francisco Bay where salt is manufactured from sea water by solar evaporation. This particular *Artemia* has been regarded as a variety of *A. salina,* and also as a separate species, *A. franciscana.* Bond (1932) suggests, after experiments on the effect of salinity on development, that it should be regarded as a separate species.

The *Artemia* from the margin of San Francisco Bay reproduce by two methods. In the presence of males the same females sometimes produce viviparous nauplii, and at other times they release encysted embryos which are encased in hard chitinous coverings or shells. These encysted embryos will not ordinarily hatch in sea water until they have first been desiccated. Air-dry cysts remain viable for many years and when they are placed in sea water, the embryos hatch as swimming nauplii.

Dry cysts are approximately ⅕ mm. in diameter. They are deeply indented on one side, but when they are placed in sea water, or in sea water of modified salinity in which they will hatch, they take up water and round out to become spherical. After a time, which depends among other things on salinity and temperature, the chitinous cyst wall or shell splits, and the embryo emerges head first encased within a delicate trans-

[1] This intolerance of potassium appears to be an important factor in the distribution of *Artemia* in desert salt lakes (Boone and Becking, 1931).

parent membrane or sac. This sac may remain attached at one end to the shell or it may at once be free. During the emergence from the shell and for some time thereafter the nauplius is quiescent within the sac. The sac finally begins to soften and dissolve and the nauplius moves its appendages. The nauplius completes its excystment by hatching or escaping from the remains of the sac, after which it swims actively about. At the time of hatching the nauplius contains an appreciable supply of yolk and even in the absence of food it develops for several days and undergoes the first moult to form a metanauplius. The external anatomy of the developmental stages is completely described and figured by Heath (1924).

The two stages or actions in the excystment, the initial emergence from the shell, and the final hatching from the membranous sac, will for convenience be referred to as "emergence" and "hatching" respectively. Both emergence and hatching proceed rapidly compared with the time lapse before emergence and between emergence and hatching. For accuracy in determining rates, it is necessary to define these two stages rather precisely even if somewhat arbitrarily. The emergence from the shell is a discreet abortive process and a nauplius is considered emerged if the eye can be seen. The first indication of hatching from the sac is usually the projection of the first pair of antennae. Soon the large second pair is also projected outside the sac and swimming or attempts at swimming begin. The first movements of the appendages are often intermittent and uncoördinated. A nauplius is considered hatched when the first two pairs of appendages project outside the sac and are motile. The third pair of appendages soon slides out and the remnant of the sac is left behind.

The effects of specific ions and of ion antagonism on the excystment of *Artemia* have been studied by Boone and Becking (1931), who also have concluded that osmotic pressure has much less effect on excystment than chemical factors. Jacobi and Becking (1933) observed that excystment will not take place in natural sea water concentrates of three or more molar equivalent. The present experiments were undertaken to test the effect of total salinity on the rate of excystment in diluted and concentrated sea water in which the proportion, or relative concentration, of the ions contained in sea water remains essentially unaltered. In the strongest concentration used (225 per cent sea water), there was no visible precipitation of any kind of salt so that the ionic proportions were unaltered except for second order differential effects on dissociation, and minor pH effects. The minimum salinity in which emergence, hatching, and early development will take place has also been determined.

Method

The Cysts

The cysts used in these experiments were generously provided through the courtesy of Dr. Alvin Seale of the San Francisco Aquarium Society. They were collected near Redwood City, California, June 10, 1937, and the experiments were carried out in the winter of 1939.

About 20 per cent of the original sample of cysts excysted in sea water. It was found by dissection that most of the remainder were empty shells of previously excysted embryos, although some contained embryos which were presumably dead. The empty shells are difficult to distinguish by simple inspection, but it was found that they could be partly separated out by rapid differential flotation since they contain air until they have soaked. The cysts were shaken and suspended in distilled water in a test tube and those that floated were discarded. Some good cysts were discarded by this method, and not all that sank were good cysts, but a stock was obtained in which the percentage which excysted had been increased from 20 to about 60. In this process the cysts were exposed to distilled water for only three minutes and were then dried on filter paper for five days at room temperature and 30 per cent humidity. After this they were stored for some time in a stoppered bottle before using. The brief washing in distilled water also served to remove most of the salt on the cysts, which is important for the present purpose. Otherwise, the differential flotation can be carried out as well in sea water (Whitaker, 1940). The rate of excystment in normal sea water varies with the duration of drying and storing after the washing, and would no doubt differ in different samples of cysts for this and other reasons. The present experiments were carried out on a single stock of cysts during a period in which the rate of excystment in normal sea water was practically constant.

The Media

Sea water (specific gravity, 1.025, pH 7.9–8.0) was collected at Moss Beach, California, and was filtered before being concentrated or diluted. The specific gravity 1.025 was taken as a base throughout and was considered to represent the salinity of what is called 100 per cent sea water. Sea water was diluted by adding triple glass distilled water to prepare the dilutions shown in Table I. It was concentrated by evaporating under reduced pressure in a water bath at 45–50° C. The resulting brine was diluted back with distilled water to prepare the salinities greater than sea water which are also shown in Table I. Specific gravities were

checked with pycnometers. The concentrating process removed gases from the brine so the solutions more concentrated than sea water were re-equilibrated by aerating for several hours with a sintered glass nozzle. A glass electrode was used to measure pH. The rate of excystment is practically unaffected by pH within the range 8.3–7.7, so that pH can hardly be an important factor in the present instance except perhaps in distilled water (see Table I).

Excystment

Small 1 cc. Syracuse dishes were used for excystment. Especially in the solutions of high salinity, the cysts tend to float and to accumulate in the meniscus where observation is difficult. Accumulation in the

TABLE I

Salinity and pH of media. For convenience in comparing, salinity is expressed as a percentage of the salinity of normal sea water (specific gravity 1.025), and a solution is described in terms of its relative salinity as the corresponding percentage of sea water.

Percentage Sea Water	Specific Gravity	pH
225	1.0562	8.0*
200	1.0500	8.2*
175	1.0438	8.3*
150	1.0375	8.3
125	1.0312	8.2
100	1.0250	8.0
75	1.0187	8.0
50	1.0125	7.9
25	1.0062	7.9
12½	1.0031	7.7
0†	1.0000	6.5

* Probably inaccurate due to effect of high salt concentration on glass electrode.
† Distilled water.

meniscus was prevented by dipping the dishes in hot, pure high melting point paraffin. A thin coating of paraffin causes the water meniscus to be inverted. In each experiment about twenty cysts were placed in 1 cc. of medium in each small dish, and ten small dishes were placed in Petri dishes arranged as moist chambers to prevent evaporation. The two most dilute solutions (0 and 12½ per cent sea water) were changed once in the course of the experiments so that the small amount of salt on the cysts would not appreciably alter the salinity. No measurable changes of salinity took place during the experiments. The moist chambers and the solutions were kept throughout in a humid constant temperature room at 25 ± ¼° C.

After emergence began in a population, counts were made of the numbers emerged and hatched at least every two hours until at least 60–70 per cent had hatched. The numbers that ultimately emerged and hatched were also determined at about 96 hours, and the original empty shells and non-viable cysts were excluded from consideration. The number emerged and the number hatched at the time of each observation were treated as percentages of the number that ultimately emerged and

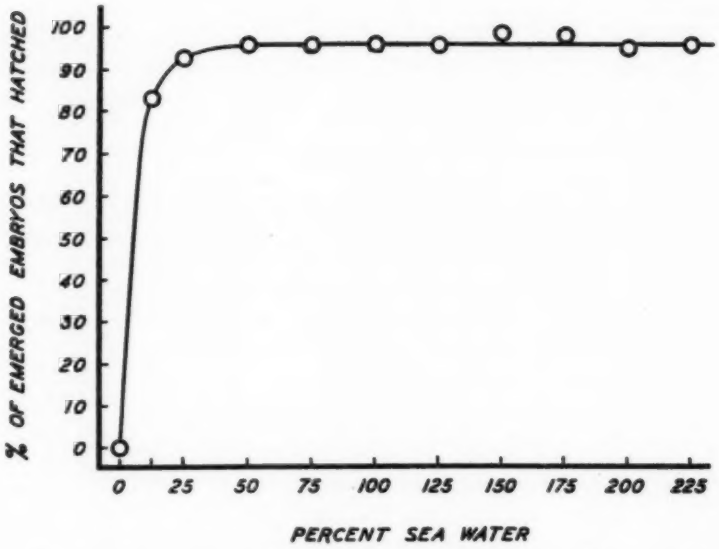

FIG. 1. The percentage of emerged embryos that hatched in diluted and concentrated sea water of various salinities. One hundred per cent sea water corresponds to specific gravity 1.025, and 0 per cent sea water is distilled water (see Table I).

hatched, respectively. The percentages obtained from successive observations were plotted against time to give signoid curves, and the times at which 50 per cent had emerged, and at which 50 per cent had hatched, were determined from these curves by interpolation.

RESULTS

Five to nine experiments (involving counts on a total of 500–1,000 viable cysts) were carried out at each salinity. Throughout the range of salinities used, and in distilled water, approximately 60 per cent of

the stock mixture of cysts and empty shells emerged (see method), i.e. all of the embryos which are presumed to have been viable emerged in all of the solutions. More than 96 per cent of the embryos that emerged from the shell also hatched from the membranous sac in 50–225 per cent sea water, inclusive, but in lower salinities this percentage decreased and no true hatching at all occurred in distilled water (Fig. 1). In distilled water the appendages of the emerged embryos did not move. The membranous sac disintegrated after several hours in about one-fifth of

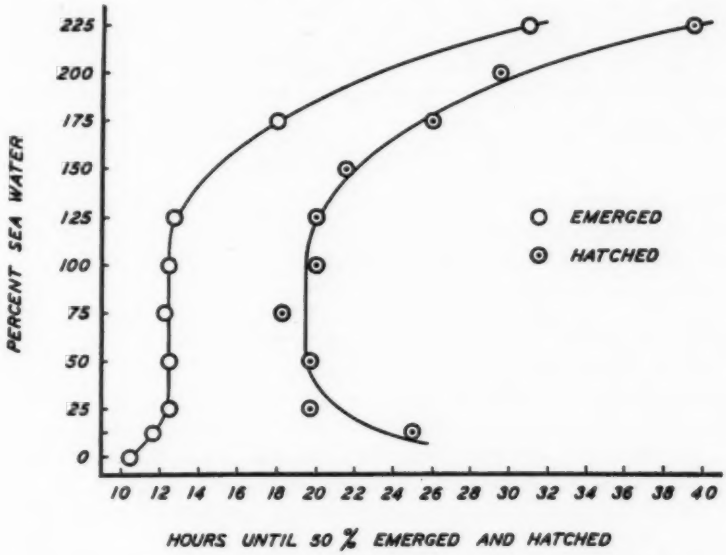

FIG. 2. The rates of emerging and hatching in diluted and concentrated sea water at 25° C. One hundred per cent sea water corresponds to specific gravity 1.025, and 0 per cent sea water is distilled water (see Table I). The curves show the time lapse until 50 per cent of the embryos in populations emerge, and until 50 per cent hatch (see text).

the cases, causing a sort of pseudo-hatching, but in these cases the nauplii always swelled, and often burst near the first joints of the large second antennae. Inactivity of the embryo probably interferes with hatching. Occasional cysts burst within 20–30 minutes after being placed in distilled water and aborted amorphous masses. This also occurred rarely in 12½ per cent sea water and in higher salinities as well.

The embryos that hatched throughout the range 12½–225 per cent

sea water were all normal, active, and viable in the salinity in which they excysted. They moulted once before dying of starvation on about the fourth day. No food was provided and no attempt was made to determine the salinity requirements of more advanced developmental stages. The effect of salinity on food organisms is a complicating factor after the yolk has been consumed.

The effect of salinity on the rate of emergence and hatching is shown in Fig. 2. Each point represents the average of the recorded time lapses until 50 per cent had emerged and until 50 per cent had hatched in the several experiments at each salinity. The results of the individual experiments were quite consistent. It may be seen in Fig. 2 that the rates of emergence and hatching are little affected by osmotic pressure within the salinity range 25–125 per cent sea water, but this is not true in higher and lower salinity.

Summary and Conclusions

1. The excystment of *Artemia* takes place in two principal stages: first, the quiescent nauplius emerges from the shell of the cyst within a membranous sac, and then later the nauplius hatches from the sac and swims actively about.

2. The excystment of *Artemia* obtained from the margin of San Francisco Bay has been studied at 25° C. in diluted and concentrated sea water over a salinity range from zero (distilled water) to 225 per cent sea water (i.e., a solution in which the salt concentration is 225 per cent of the salt concentration of sea water. No salts precipitated out).

3. The same percentage of embryos emerged from the shells in all of these salinities, including zero salinity (distilled water).

4. In distilled water the emerged embryos are motionless and they do not hatch from the sac. Some swell and burst.

5. In 12½ per cent sea water, 83 per cent of the emerged embryos hatch; in 25 per cent sea water 93 per cent hatch. In 50–225 per cent sea water 96–99 per cent hatch.

6. In 12½–225 per cent sea water the nauplii that hatch are normal, active, and viable. They moult to form metanauplii before dying of starvation (in the absence of food) on about the fourth day.

7. In the salinity range 25–125 per cent sea water, the rates of emergence and hatching are practically constant (and therefore independent of change in osmotic pressure).

8. In the salinity range 150–225 per cent sea water the rates of emerging and hatching decrease with increasing salinity, but the interval between emergence and hatching is nearly constant throughout the range 25–225 per cent sea water.

9. Emergence is accelerated in 12½ and 0 per cent sea water, but hatching is retarded in 12½ per cent sea water and is inhibited in distilled water.

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SPERM ACTIVATION BY ARBACIA EGG EXTRACTS, WITH SPECIAL REFERENCE TO ECHINOCHROME

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It has long been known that the eggs of various marine invertebrates secrete substances which markedly affect the behavior of sperm. One has merely to rinse eggs in sea water and add this water to a sperm suspension to produce striking changes. These effects are classified by Lillie (1924) as activation, aggregation, and agglutination. Activation is a stimulation of the sperm, bringing them instantaneously from an inactive state (as in the testis) to a high pitch of activity. Aggregation constitutes the gradual accumulation of the sperm within a region of high concentration of various agents, and in the case of egg-secretions, appears to be a chemotaxis. Agglutination is the clumping of the sperm exposed to egg-secretions. While some properties of the substances active in egg-secretions are known (Tyler and Fox, 1940), the substances themselves have not been isolated from eggs in pure enough form that we can attribute these properties to definite chemical entities. In 1939, Hartmann, Schartau, Kuhn, and Wallenfels reported that echinochrome, the pigment which gives *Arbacia* eggs their reddish color, produces the same stimulation in *Arbacia pustulosa* sperm as does the egg-secretion itself, and is effective in dilutions as great as 1 : 2,000,000,000. An attempt was made to duplicate these results, using *Arbacia punctulata* sperm, and crystalline echinochrome kindly supplied by Dr. E. G. Ball, which he had isolated from *A. punctulata* eggs (1934). No stimulating effect could be detected. Subsequently there appeared a fuller account by Hartmann and Schartau (1939), and a report by Tyler (1939) of negative results with *Strongylocentrotus purpuratus*. Because of this, it seemed worthwhile to repeat and extend the experiments with *A. punctulata*.

Echinochrome as the Activator

In preparing the solutions, both sea water and isotonic sodium chloride were used. In sodium chloride, the sperm do not agglutinate, which

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sometimes facilitates comparison of sperm activity. The egg-secretions were obtained by suspending one volume of eggs in 100 volumes of sea water (or NaCl) for a half-hour. The liquid, filtered free of eggs, is generally referred to as egg-water, and is extremely effective in bringing about activation and agglutination. The concentrations of echinochrome ranged from 1:2,500,000,000 to 1:25,000. At the latter concentration, the echinochrome solution is pink in color and therefore is well above the concentration of echinochrome in active egg-water, which was colorless. The activating properties of the echinochrome solutions were tested immediately after preparation, to avoid possible loss of the echinochrome through its precipitation as a calcium salt in sea water, or its decomposition in alkaline solutions.

Sperm respond readily to differences in pH. Therefore, in testing potency of echinochrome, particular attention was given to the control of pH in all solutions used. Glycyl-glycine (.001 M) and piperazine (.001 M) were used as buffers in preference to phosphate, which tends to precipitate calcium and magnesium from the sea-water solutions (Tyler and Horowitz, 1937). In these experiments little strain is placed upon the buffer systems, and measurements with the glass electrode showed these low concentrations to be adequate. In any single series, the egg-water and echinochrome solutions, and the sperm suspensions were prepared with the same buffer and the pH was measured with a glass electrode before and after the activation tests were made, as further precaution against differences in hydrogen ion concentration. Most of the tests were carried out at a pH level where the sperm were inactive, but were readily activated when egg-water at the same pH was added. This pH value was found to be in the neighborhood of 6.0 for the sea water and 7.5 for the isotonic sodium chloride solutions. The actual pH values varied with the individual sea-urchin, and increased if the dry sperm was allowed to age. In these experiments the absolute value is not important, since in every case a control test with egg-water was made along with each test of echinochrome. Accordingly, if echinochrome is the activating agent in egg-water, it should show activating properties at the same pH as the egg-water.

Two methods of testing were employed. In one, the dry sperm, that is, the sperm taken directly from the testis with a minimum of moisture, was diluted to about 1:100 in buffered sea water or isotonic NaCl. A drop of this was covered with a cover-glass, and the egg-water and echinochrome pipetted into opposite sides of the drop. In this way the slightest response of the sperm could be detected and a precise comparison made between the two solutions. Adding dry sperm directly to the test solution sometimes gave more spectacular differences in re-

sponse, but where small differences are involved, the first method is freer from subjective interpretation.

In no case did echinochrome activate the sperm. Each test was accompanied by a test with egg-water at the same pH, in which activation did occur.

Additional tests were carried out at higher pH values to supply more nearly normal conditions for the sperm. The results are not as clear-cut as with inactivated suspensions, since differences in speed of sperm are hard to estimate. However, in no case could it be said that the echinochrome definitely produced an increase in motility greater than did mere dilution with buffered sea water, whereas stimulation by egg-water could usually be seen clearly.

Chemotaxis in Echinochrome

To check the reported chemotactic effect of echinochrome, a few tests were made to compare the migration of sperm up glass capillary tubes. Tubes of the same diameter were washed and filled with sea water, egg-water, and echinochrome solution. The ends were then inserted into buffered sperm suspension, and migration measured at various times. The results were so variable that none of the solutions could be said to be definitely chemotactic on the basis of these few trials. Variations in the alkalinity of the glass probably played some part, since the volume of solution was small in proportion to the surface of the tube, and the buffer capacity of the solutions was low. Under such conditions a shift toward alkalinity could occur and give an illusory chemotactic effect by merely speeding the progress of the sperm.

Activity of the Echinochrome-protein Complex

The complex which echinochrome forms with proteins from the *Arbacia* egg was reported by Kuhn and Wallenfels (1940) to have sperm-stimulating properties in even greater dilutions (1 : 300,000,000,000) than echinochrome alone, in uncombined form. A similar echinochrome complex was extracted from *Arbacia punctulata* by their method. Eggs were frozen, crushed, and extracted with sea water. After filtering, an equal amount of saturated ammonium sulphate solution was added, bringing down a rose-colored precipitate which redissolved in sea water without leaving any residue. It was purified by repeated precipitation, centrifugation, and decantation.

This complex, buffered and tested in the same way as echinochrome, both activated and agglutinated *punctulata* sperm. In biological properties and solubility, therefore, it is the same as the tertiary complex ob-

tained by Kuhn and Wallenfels. In view of the tests with pure echinochrome, however, the activity of the complex would seem to be centered in the protein moiety rather than in the echinochrome.

Separation of the Agglutinating and Activating Properties of Egg-water

To determine more of the nature of the activating agent, egg-water was dialyzed against sea water for a half-hour, then both fractions buffered and tested for activity. The dialysate stimulated without agglutinating, while the residue both stimulated and agglutinated sperm. Dialysis, then, can separate the agglutinating and activating agents. Distillates obtained by gently boiling each fraction also showed activating properties, but the activity disappeared shortly after the distillate was buffered to pH 6. The original, unboiled dialysate retained its activity 24 hours, beyond which it was not tested. Similarly, a distillate from a repeatedly precipitated and washed sample of the echinochrome-protein complex could stimulate, when unbuffered (pH 9.0), and lost its activity within an hour after it was buffered to pH 6.6. This disappearance of stimulating activity from the distillates suggests that the activating agent had been altered during distillation. Improved methods of separation will probably yield a stable stimulating fraction. At present, it is important that distillates of egg-water, of egg-water dialysate, and of the echinochrome complex are similar in that they contain an activating substance.

Discussion

The absence of visible response of *A. punctulata* sperm to echinochrome is in agreement with Tyler's investigations with *Strongylocentrotus purpuratus*. He found that echinochrome brought about no increase in oxygen consumption of the sperm or eggs. On the other hand, these results do not agree with the observations of Hartmann and Schartau on *A. pustulosa*, which was found to be extremely sensitive to echinochrome solutions. The difference in response of *A. pustulosa* and *A. punctulata* could be attributed to species difference, although this would make the similarity of *A. punctulata* to the more distantly related *Strongylocentrotus* appear somewhat anomalous. Another possibility is that Hartmann and Schartau did not control pH in their solutions, since it is not mentioned in any of the papers on *A. pustulosa*. Their results, particularly the activity of highly dilute solutions (1:2,500,000,000), suggest that the activation is due to the normal alkalinity of the sea water used as a solvent.

Whatever the final answer may be with regard to echinochrome, we

must search farther for the answer to the general problem of sperm-activation by egg-secretions. Echinochrome is limited in occurrence, even within the class *Echinoidea*. Moreover, the egg-secretions from pigmented eggs will stimulate sperm from unpigmented species, and vice versa (cf. Woodward, 1918, Table I).

In the *Arbacia* egg there is some sperm-activating substance which will dialyze through a collodion membrane. It can, then, be separated from the agglutinating substance with which it is closely associated, but which will not dialyze. However, the echinochrome-protein complex carried with it through seven precipitations the power to activate as well as agglutinate sperm. In view of the ease with which the activator dialyzes, one might well expect it to be washed completely free from the agglutinating substance. Tyler (1939) also reports that partial purification of agglutinin from the keyhole limpet does not free it from activating properties. This leads one to suspect that there may be two substances present which activate sperm: one closely attached to the agglutinating substance, and one easily separated from it. On the other hand, the activating properties of rough distillates from the egg-water dialysate and the partially purified echinochrome-protein complex is some evidence of similarity between the activating agents in both, but it does not prove identity.

Earlier work on extracts from echinoderm eggs offers a possible explanation of these observations. Woodward (1918) obtained, in addition to an ammonium sulphate precipitate of agglutinin, a barium chloride precipitate which showed lipolytic activity. Glaser (1921) pointed out that this lipolysin (and pancreatic lipase as well) could activate sperm. Yet, if this lipase is the activating agent in egg-water, it follows that the activator in the distillates must be some substance other than the lipase, since a protein would not distil. In this respect the work of Clowes and Bachman (1921) takes on added significance. They were not only the first to obtain sperm-activating distillates from egg-water, but also found that higher alcohols (propyl, allyl, and cinnamyl) and related substances activate sperm. Bringing these findings together, one might tentatively suggest that the immediately effective agent in sperm activation is an alcohol freed by the lipase. Then the presence of the alcohol or of the lipase would be adequate to produce sperm activation. It remains to be demonstrated that the sperm-activator that follows the agglutinating fraction is the lipase, and the dialysable, distillable activator is the product of the activity of that lipase. The hypothesis fits the framework of assembled facts, but substantiation will require considerable further investigation of egg-water fractions and egg extracts.

SUMMARY

Echinochrome is not the agent in *A. punctulata* egg-water which stimulates sperm. The echinochrome-protein complex precipitated from the extract of crushed eggs by $(NH_4)_2SO_4$ is an effective sperm stimulator. From egg-water dialysate and from the echinochrome-protein complex a distillate can be obtained which has sperm-activating properties. It is tentatively suggested that a higher alcohol freed by a hydrolytic agent in the egg-water is the stimulating substance acting directly upon the sperm.

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THE NUTRITIONAL REQUIREMENTS OF TRIBOLIUM CONFUSUM DUVAL

I. The Survival of Adult Beetles on Patent Flour and Complete Starvation Diets [1]

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Introduction

The attention of numerous workers has recently been drawn to the problem of determining the nutritional requirements of insects. The problem has been attacked along two general lines of procedure, namely: (1) by observations on the ability of an organism to survive on a given diet; and (2) by analyses of the different regions of the digestive tract of the organism to determine the presence or absence of the various digestive enzymes. The present series of studies on *Tribolium* concerns the former aspect of the problem. The importance of determining the nutritional requirements of *Tribolium* was emphasized in an earlier paper (Schneider, 1940) on thyroid feeding. There it was stated that "When the fields of the nutritional requirements and the endocrinology of insects have been thoroughly worked, we shall be in a much better position to determine the effect of a given vertebrate hormone extract on a given insect than we are at present."

Chapman (1924), Sweetman and Palmer (1928), Street and Palmer (1935), Nelson and Palmer (1935), Bushnell (1938), and Chiu and McCay (1939), all working with *Tribolium*, studied the nutritional requirements with respect to group biology or population growth and maintenance. Since there are many factors in addition to the nutritional quality of the food medium which affect population growth, i.e., population density, cannibalism, and "conditioning" of the food medium (Park, 1934*a*), it seemed expedient to study the problem from a more fundamental viewpoint, namely that of determining the individual performances of isolated beetles fed on various diets. From logical considerations it appears that an approach to the problem from this point of

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view is likely to lead to a sounder foundation of the nutritional requirements of insects *per se*, than can be attained from following any other line of investigation.

The present investigation has indicated that the starvation control is of considerable value in nutritional studies, and is particularly desirable in this case in view of the long life (up to three years) of the organism on the so-called "normal diet," flour. The term "normal diet" is here used in the sense that the beetles have been found to grow and reproduce in it, though it may not really be adequate for optimal physiological processes. The value of a particular diet can be determined just as accurately by comparison of the performances of the animals fed on it with those of animals on complete starvation, as it can by comparison with those of animals fed the "normal diet," and in this particular case it is certainly less time-consuming. In other words, if we start with the starvation diet as the zero point on a scale, all other diets, including the present so-called "normal diet" can be referred to the zero point in terms of either positive or negative values, i.e., they will be either better or worse than no food at all.

Statement of the Problem

Pearl and Parker (1924) have stated that ". . . when we study duration of life under normal conditions we are dealing with the combined effects of two variable complexes, inborn organization, on the one hand, and environment, including renewal of available energy and substance by food, on the other hand." The former complex, inborn organization, designated by Ashby (1930) as the "capital" of the organism, has also been termed the inherent vitality of the organism. Since the knowledge of the inherent vitality of *Tribolium* is primarily essential to the knowledge of its general nutritional requirements, the present investigation has been designed to deal principally with this phase of duration of life. Hence, the problem may be stated as that of determining the ability of the adult beetle, *Tribolium confusum* Duval, to survive under conditions of complete starvation. Several more specific problems dealing with the influence of various environmental factors of pre-imaginal life on the ability of the adult beetle to survive conditions of complete starvation are involved.

Technique

Park (1934*b*) has described in detail the general technique for handling *Tribolium* cultures in the laboratory, and there is no need for its repetition here. It is sufficient to state that the beetle spends its entire life history in flour, and by the use of sieves of various sized mesh, the

adults, eggs, and all immature forms can be separated from the flour for observation. The sex is determined from the pupal characteristics.

Throughout the present investigation, when eggs were used to start an experiment, a sufficient number was taken from a general stock culture, and allowed to hatch in dishes containing a small quantity of patent flour (Ceresota). The date of hatching was determined within 0.5 day, and the larvae were placed in bottles in a definite quantity of flour (indicated in the experiments). The bottles were kept in a darkened constant temperature incubator at a temperature of 29° C., and a relative humidity of approximately 40 per cent. The food was not changed, and the bottles were not sifted until about the twentieth day after hatching, when it became necessary to sift them once a day to collect pupae. The date of pupation was recorded to the nearest 0.5 day (the color of the pupa indicating whether the pupal stage was reached within the past 0.5 or 1.0 day). The pupae were sexed and placed in sterile vials (1.5 cm. diam. $\times$ 2.5 cm. tall) and kept in the incubator under the above-mentioned physical environmental conditions. The date of emergence was recorded to the nearest 0.5 day, and the discarded pupa cases were removed from the vials. The vials were closed with corks covered with cellophane, the corks having a filed groove along the side, and the cellophane being perforated so as to maintain a normal oxygen supply within the vials. The experience of the writer in having beetles escape by eating their way through the cork along the filed groove prompted the use of the cellophane covering. This prevented the boring activity in all but a very few vials where the cellophane was broken, and of course these few cases were immediately discarded. The date of death was recorded to the nearest day, and the period from emergence to death (adult survivorship) was calculated.

When only adults of a known age and sex were needed for an experiment, it was necessary to start by collecting pupae from a general or specific stock culture (indicated in the experiments). These were sexed and placed in vials, from which point the procedure was the same as that described above.

In the one experiment where flour-fed controls were used, the vials were supplied frequently with plenty of fresh flour, so that the organism was always presented with an unlimited quantity of food. Data on the weights of pupae, live and dead imagos, have been reserved for a later publication.

The statistical data are based on frequency distributions of the variables studied. The $\frac{\text{Diff.}}{\text{P. E. Diff.}}$ test for significance was used; any value higher than 3 indicating that the difference was probably not due to sampling errors.

Observations and Results

Survivorship of Adult Beetles, Taken as Pupae from a General Stock Culture and Subjected to Flour and Starvation Diets upon Emergence

In the first experiment 432 pupae (216 of each sex) were isolated from a general laboratory stock culture, and were observed daily for emergence. Since these forms were taken from a general stock culture, they were reared under identical, though unknown factors of larval density. Upon emergence, 100 of each sex were submitted to conditions of complete starvation, and 116 of each sex were given a flour diet. Daily observations were made for mortality, and the time of death was recorded. The time intervening between emergence and death was calculated as adult survivorship. Failure of the organism to respond to agitation with a small camel's hair brush was taken as the criterion for death.

Table I presents the data relative to the survivorship of these two groups of organisms.

TABLE I

Survivorship of adult beetles on flour and starvation diets.

Group	Range		Mean number days of life at end of 24 days	Median number days of life at end of 24 days	Standard deviation (days)	Coefficient of variation (per cent)	*N*
	Min. (days)	Max. (days)					
Starved adult males....	2.5	21.0	14.235±0.274	14.665±0.343	4.062±0.194	28.53±1.46	100
Flour-fed adult males...	2.5	24.0	21.323±0.370	24.181±0.464	5.908±0.262	27.70±1.32	116
Starved adult females...	3.0	23.5	13.535±0.314	14.000±0.394	4.648±0.222	34.34±1.82	100
Flour-fed adult females.	2.5	24.0	22.964±0.240	24.221±0.301	3.826±0.169	16.16±0.76	116
Total starved adults....	2.5	23.5	13.885±0.213	14.593±0.267	4.459±0.150	32.11±1.19	200
Total flour-fed adults...	2.5	24.0	22.166±0.220	24.202±0.276	4.974±0.156	22.43±0.74	232

The data are presented for the sexes separately and then combined. Since all the beetles on starvation were dead at the end of 24 days, it appeared justifiable to compare their survivorship performances with those of the flour-fed group up to the end of 24 days. This procedure obviated the necessity of observing the flour-fed group for their entire life (which may have been up to three years). For a study of the complete survivorship record of flour-fed *Tribolium,* the reader is referred to Pearl, Park and Miner (1941). In the present case it was not possible to calculate mean length of life of the flour-fed group; instead there is calculated the mean number of days of life lived up to the end of 24 days per beetle exposed to risk.

The difference between the mean length of life of the starved beetles (13.885 ± 0.213 days) and the mean number of days of life at the end

TABLE II

Ungraduated life table for flour-fed and starved adult Tribolium.

Day of age	Flour-fed												Starved											
	Male				Female				Both sexes together				Male				Female				Both sexes together			
	Number living at start of age period	Deaths	Death rate per 1000	Survivors per 1000	Number living at start of age period	Deaths	Death rate per 1000	Survivors per 1000	Number living at start of age period	Deaths	Death rate per 1000	Survivors per 1000	Number living at start of age period	Deaths	Death rate per 1000	Survivors per 1000	Number living at start of age period	Deaths	Death rate per 1000	Survivors per 1000	Number living at start of age period	Deaths	Death rate per 1000	Survivors per 1000
0– 1	116	0	0	1000	116	0	0	1000	232	0	0	1000	100	0	0	1000	100	0	0	1000	200	0	0	1000
1– 2	116	0	0	1000	116	0	0	1000	232	0	0	1000	100	0	0	1000	100	0	0	1000	200	0	0	1000
2– 3	116	3	26	1000	116	0	0	1000	232	3	13	1000	100	3	30	1000	100	1	10	1000	200	4	20	1000
3– 4	113	2	18	974	116	1	9	1000	229	3	13	987	97	1	11	970	99	3	30	990	196	4	20	980
4– 5	111	0	0	957	115	2	17	991	226	2	9	974	96	2	21	960	96	3	31	960	192	5	26	960
5– 6	111	0	0	957	113	0	0	974	224	0	0	966	94	1	11	940	93	5	54	930	187	6	32	935
6– 7	111	2	18	957	113	0	0	974	224	2	9	966	93	2	22	930	88	3	34	880	181	5	28	905
7– 8	109	4	37	940	113	0	0	974	222	4	18	957	91	1	11	910	85	3	35	850	176	4	23	880
8– 9	105	2	19	905	113	2	18	974	218	4	19	940	90	4	44	900	82	1	12	820	172	5	29	860
9–10	103	0	0	888	111	0	0	956	214	0	0	922	86	2	22	860	81	6	74	810	167	8	48	835
10–11	103	0	0	888	111	0	0	956	214	0	0	922	84	1	12	840	75	5	67	750	159	6	38	795
11–12	103	1	10	888	111	0	0	956	214	1	5	922	83	2	24	830	70	3	43	700	153	5	33	765
12–13	102	1	10	879	111	0	0	956	213	1	5	918	81	6	74	810	67	9	134	670	148	15	101	740
13–14	101	1	10	871	111	0	0	956	212	1	5	913	75	12	160	750	58	8	138	580	133	20	150	665
14–15	100	1	10	862	111	0	0	956	211	1	5	909	63	21	333	630	50	8	160	500	113	29	257	565
15–16	99	0	0	853	111	0	0	956	210	0	0	905	42	12	286	420	42	10	238	420	84	22	262	420
16–17	99	1	10	853	111	0	0	956	210	1	5	905	30	12	400	300	32	10	303	320	62	22	355	310
17–18	98	2	20	845	111	2	18	956	209	4	19	901	18	5	278	180	22	5	227	220	40	10	400	200
18–19	96	1	10	827	109	1	9	939	205	2	10	884	13	6	462	130	17	8	471	170	30	14	466	150
19–20	95	2	21	819	108	1	9	931	203	3	15	875	7	4	571	70	9	4	444	90	16	8	500	80
20–21	93	0	0	802	107	2	19	922	200	2	10	862	3	3	1000	30	5	1	200	50	8	4	500	40
21–22	93	0	0	802	105	0	0	905	198	0	0	853	0	0	—	0	4	3	750	40	4	3	750	20
22–23	93	1	9	802	105	1	10	905	198	2	10	853					1	1	1000	10	1	1	1000	5
23–24	92	1	9	793	104	0	0	896	196	1	5	845					0	0	—	0	0	0	—	0
24–25	91	0	0	784	104	0	0	896	195	0	0	841												

of 24 days of the flour-fed group (22.166 ± 0.220 days) is 8.281 ± 0.306 days ($27.06 \times$ P. E.). The sex difference, though not a significant one in the case of the starved forms, indicates that the males on the average survived slightly longer than the females. The opposite is true for the flour-fed group, i.e., the females showed a higher mean number of days of life at the end of 24 days than the males. The latter difference amounts to 1.641 ± 0.441 days ($3.72 \times$ P. E.).

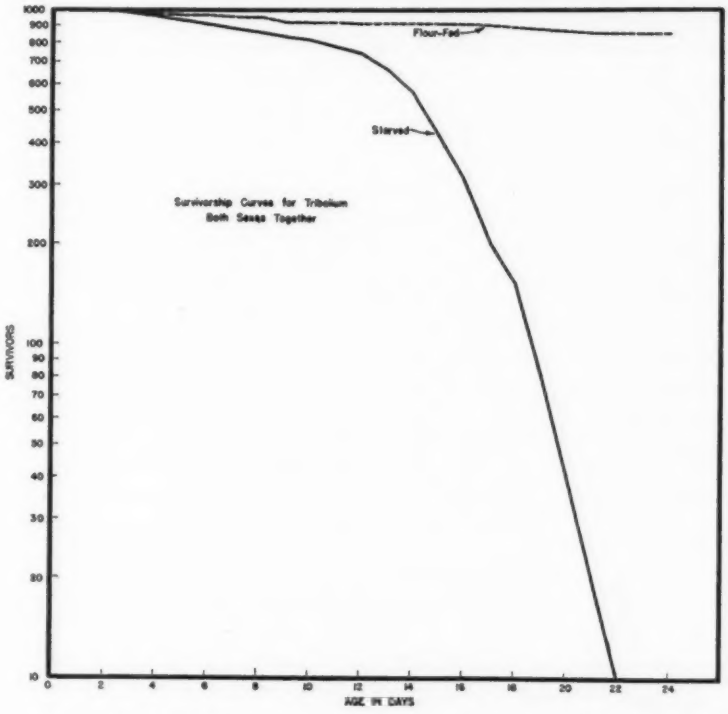

FIG. 1. Survivorship curves for the flour-fed and starved adults of *Tribolium*, taken as pupae from a general stock culture. Flour-fed: $N = 232$; observed 24 days. Starved: $N = 200$; observed entire life.

Table II is the ungraduated life table for these two groups of organisms. The four columns under each sex heading show: (1) the absolute number of beetles living at the beginning of each day of age and therefore exposed to the risk of dying during that day; (2) the number of deaths actually occurring during each day of age; (3) death

rate for each day of age per 1000 living at the beginning of the day; and (4) survivors per 1000 exposed to risk at the beginning of each day of age.

It is true that a few females of the starved group lived longer than any males of that group, but the fact that a greater number of males survived through the 12- to 14-day period accounts for the higher average survivorship of the males. In the flour-fed group the females took an early lead in survivorship and maintained that lead at an increasing

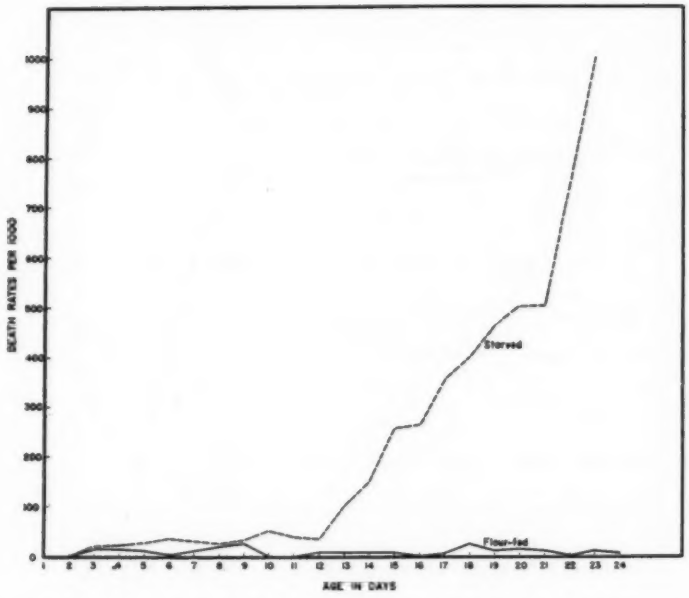

FIG. 2. Observed (ungraduated) death rates (deaths per 1000 exposed to risk) for flour-fed and starved adult *Tribolium*, taken as pupae from a general stock culture.

rate to the end of the 24-day period of observation. Figure 1 shows the survivorship curves for the starved and flour-fed groups, sexes combined.

The only other factor of importance in discussing this particular experiment is the mortality rate. This may be studied from the life table for these two groups of beetles (Table II) and from the mortality curves presented in Fig. 2.

From Fig. 2 it is evident that the mortality rates exhibited no great differences between the flour-fed and starved groups of organisms up to the twelfth day, though during this time the rate for the starved group was slightly higher, on the whole, than that for the flour-fed group. After the twelfth day the mortality rate of the starved beetles went up very rapidly, while that of the flour-fed group continued to fluctuate just a little above the zero line. The point of interest here is that starving the adult beetle for the first 10 or 12 days after emergence does not seriously affect the rate of mortality. If starvation is continued beyond this point, however, the necessity for an exogenous supply of matter and energy for the maintenance of normal physiological processes manifests itself in a sudden rise in the rate of mortality. Whether or not the adult beetle will recover from the effects of starvation if returned to a diet of flour on the twelfth day is a problem for further investigation.

An observation worthy of note here is that the starved beetles produced fecal pellets throughout their entire lives.

The question may be raised as to whether the starved female adults may not have laid eggs and eaten them, and hence may not represent a completely starved group. To answer this point, it can first be stated with certainty that no eggs were ever seen in the vials containing the starved females. This means that the females either ate the eggs immediately after laying them, or that they never laid any eggs at all. Further experimental evidence points to the latter conclusion as the correct one. Virgin females from the flour-fed group were observed carefully for daily egg-laying. In no case were any eggs found under 12 days. At the end of 20 days the cumulative egg-laying record of 185 virgin females in flour was as follows:

49 females had laid 0 eggs at the end of 20 days.
29 females had laid 1 egg at the end of 20 days.
22 females had laid 2 eggs at the end of 20 days.
20 females had laid 3 eggs at the end of 20 days.
21 females had laid 4 eggs at the end of 20 days.
8 females had laid 5 eggs at the end of 20 days.
6 females had laid 6 eggs at the end of 20 days.
13 females had laid 7 eggs at the end of 20 days.
4 females had laid 8 eggs at the end of 20 days.
2 females had laid 9 eggs at the end of 20 days.
3 females had laid 10 eggs at the end of 20 days.
2 females had laid 11 eggs at the end of 20 days.
3 females had laid 12 eggs at the end of 20 days.

It is clear that over 76 per cent of these beetles had laid fewer than 5 eggs each at the end of 20 days.

From this sample of 185 virgin, flour-fed females, it is evident that in the absence of the male, the beginning of egg-laying is decidedly retarded (mated, flour-fed females begin laying 2 or 3 days after emergence). This fact, as well as the low mean length of life of starved females, in addition to the poor body nutrition of the starved group at the time when they would have started laying, all point strongly to the probability that the starved females never laid any eggs during their lives. Furthermore, the fact that the mean length of life of the starved females was slightly less than that of the starved males indicates that even if the females did lay a few eggs and eat them, this action did not contribute greatly to their supply of nutrition.

TABLE III

Length of larval period for various larval population densities.

Larval density	Range		Mean length of larval period (days)	Median length of larval period (days)	Standard deviation (days)	Coefficient of variation (per cent)	*N*
	Min. (days)	Max. (days)					
(*a*) 100 larvae per 100 grams flour	25.5	40.0	34.095±0.106	33.431±0.133	2.719±0.075	7.97±0.22	297
(*b*) 10 larvae per 10 grams flour	25.5	52.0	35.922±0.149	35.281±0.187	3.726±0.105	10.37±0.29	285
(*c*) 300 larvae per 100 grams flour	29.5	64.0	37.460±0.229	36.038±0.287	5.642±0.162	15.06±0.44	276

It is evident that when an experiment such as this is started with pupae from a general stock culture, nothing is known of the population factors under which the larvae developed. If pupae are taken from various stock cultures where larval density factors are almost certain to be unequal, and if larval density does have an influence on adult inherent vitality, then it is clear that the experiment has not been well controlled. In the present experiment, the pupae were all taken from the same stock culture, and hence developed as larvae under identical conditions, though these conditions were probably not optimal. In order to test the influence of larval density on adult inherent vitality, and to find the larval density conducive to the greatest adult vitality, further experiments were made.

The Influence of Larval Density on Inherent Vitality of Adult Tribolium

Larval Development.—For this investigation, eggs were collected from a general laboratory stock culture of *Tribolium*, and allowed to

hatch in a small quantity of flour. Every 12 hours the newly-hatched larvae were collected, and placed in various sized bottles under three different food-density relationships. Each group contained 300 larvae and the density relationships were as follows:

(*a*) 100 larvae in 100 grams of flour.
(*b*) 10 larvae in 10 grams of flour.
(*c*) 300 larvae in 100 grams of flour.

The larvae in (*b*) were kept in 1-ounce bottles; those in (*a*) and (*c*) in half-pint milk bottles. Not only were the survivorship performances of the resulting adults compared for the three series, but also the developmental periods for the larvae.

Table III presents data relative to the length of time spent in the larval stage for the three larval population densities.

From Table III it is evident that the larvae in density (*a*) developed in less time than those from either (*b*) or (*c*). Comparing the mean length of larval period for group (*a*) with that of group (*b*) and (*c*), respectively, we find differences of 1.827 ± 0.183 days ($9.98 \times$ P. E.) and 3.365 ± 0.252 days ($13.35 \times$ P. E.). Each difference is a statistically significant one, and each points to a more rapid larval development of the (*a*) group. Comparing (*b*) and (*c*), the difference of 1.538 ± 0.273 days ($5.63 \times$ P. E.) is also a statistically significant one, and indicates a more rapid rate of development for the (*b*) than for the (*c*) group. From the standpoint of larval development, density (*a*) produced the most rapid rate; density (*b*) the next most rapid rate; and density (*c*) the slowest rate. These observations are substantially in accord with those of Park (1938). The standard deviations indicate that the variation in length of larval period is positively associated with the absolute length of larval period; i.e., with an increase in mean length of larval period comes an increase in the amount of variation. This fact may be grasped more quickly by reference to Fig. 3.

The curve representing density (*a*) is high and narrow; that for density (*c*) is low and broad, tailing off far to the right; while that for density (*b*) falls between these two extremes. Not only is the absolute variation greater for the greater length of larval period, but also the variation relative to the mean (coefficient of variation) as indicated in Table III.

Of the 300 larvae which were started in each series 297 reached pupation in density (*a*), 285 in density (*b*), and 276 in density (*c*), indicating that the larval mortality was influenced by the density factor also. The percentage mortality for the three groups was as follows: (*a*) 1.00 ± 0.57 per cent; (*b*) 5.00 ± 1.26 per cent; and (*c*) $8.00 \pm$

1.57 per cent. Comparing these percentages we find the following differences: (*a*) vs. (*b*), 4.00 ± 1.38 per cent (2.90 × P. E.); (*a*) vs. (*c*), 7.00 ± 1.67 per cent; (4.19 × P. E.); and (*b*) vs. (*c*), 3.00 ± 2.01 per cent (1.49 × P. E.). Only one of these differences is statistically significant; that existing between groups (*a*) and (*c*). Clearly something in addition to chance fluctuation caused the heavier mortality in group (*c*). In all probability, it was the larval density factor which was responsible for this effect. The difference between (*a*) and (*b*) (2.90 × P. E.) is a borderline case of significance, and hence cannot be considered a real difference. The difference between (*b*) and (*c*) is

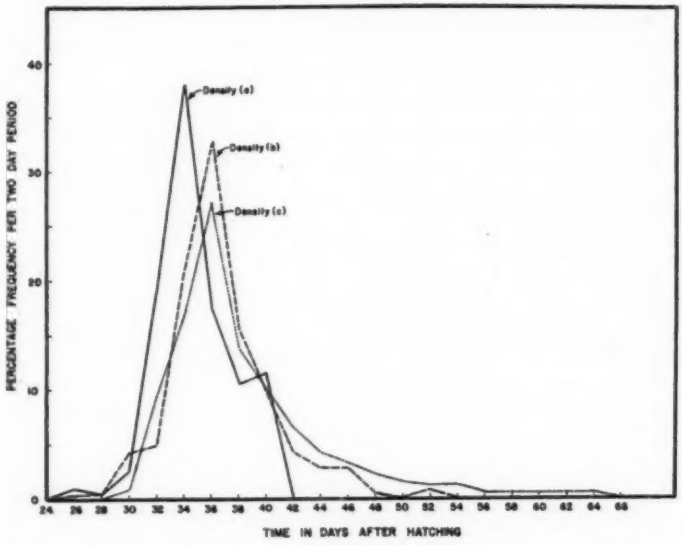

FIG. 3. Frequency distribution of length of larval period for various larval population densities.

clearly not significant. These observations on the density factor as related to larval mortality are also in accord with those of Park (1938).

It is easy to understand the differences that exist between densities (*a*) and (*c*), since there are three times as many larvae per unit of environment in (*c*) as there are in (*a*). However, the differences between (*a*) and (*b*) are more difficult to fathom in view of the fact that the two had the same population density per unit of environment. The only difference lay in the size of the total populations; one was made up of 10 larvae in 10 grams of flour, the other of 100 larvae in 100 grams

of flour. Just why the 100/100 condition was conducive to a lower larval mortality, and more rapid larval development than the 10/10 is not apparent at the moment, though the phenomenon is certainly worthy of further investigation.

Adult Survivorship.—After reaching the adult stage, the organisms from the three larval densities described above were isolated and subjected to complete starvation. The survivorship (period from emergence to death) data for the sexes separately, then combined for the three experimental groups are presented in Table IV.

There was only a small and statistically insignificant difference in survivorship between the sexes in densities (*a*) and (*c*). In density

TABLE IV

Length of life of starved adults, reared under various larval population densities.

Larval density	Range		Mean length of life (days)	Median length of life (days)	Standard deviation (days)	Coefficient of variation (per cent)	*N*
	Min. (days)	Max. (days)					
Male							
(*a*) 100 larvae per 100 grams flour	1.5	25.0	18.739 ± 0.118	19.353 ± 0.148	2.086 ± 0.083	11.13 ± 0.45	142
(*b*) 10 larvae per 10 grams flour	3.0	23.0	16.086 ± 0.159	16.500 ± 0.199	2.887 ± 0.112	17.94 ± 0.72	150
(*c*) 300 larvae per 100 grams flour	3.5	21.5	17.007 ± 0.154	17.568 ± 0.193	2.632 ± 0.109	15.47 ± 0.65	133
Female							
(*a*) 100 larvae per 100 grams flour	3.0	24.0	18.753 ± 0.173	19.375 ± 0.217	3.169 ± 0.122	16.89 ± 0.67	152
(*b*) 10 larvae per 10 grams flour	8.0	23.5	17.088 ± 0.061	17.202 ± 0.076	1.018 ± 0.043	5.96 ± 0.26	125
(*c*) 300 larvae per 100 grams flour	4.0	22.0	17.547 ± 0.169	18.333 ± 0.212	2.918 ± 0.119	16.62 ± 0.70	136
Total							
(*a*) 100 larvae per 100 grams flour	1.5	25.0	18.763 ± 0.128	19.393 ± 0.160	3.251 ± 0.090	17.32 ± 0.49	294
(*b*) 10 larvae per 10 grams flour	3.0	23.5	16.414 ± 0.105	17.046 ± 0.131	2.593 ± 0.074	15.79 ± 0.46	275
(*c*) 300 larvae per 100 grams flour	3.5	22.0	17.181 ± 0.115	18.032 ± 0.144	2.794 ± 0.081	16.26 ± 0.48	269

(*b*) this difference is 1.002 ± 0.170 days ($5.88 \times$ P. E.). In all three cases, however, the differences indicate that the females survived longer than the males. For the combined sexes, there are large differences in survivorship between the adults reared as larvae, under different population densities. Group (*a*) showed the best survivorship; group (*c*) the next best; and (*b*) the worst. A comparison of the mean length of adult life of group (*a*) with that of group (*b*) and (*c*) respectively, reveals differences of 2.349 ± 0.166 days ($14.15 \times$ P. E.) and 1.582 ± 0.172 days ($9.20 \times$ P. E.). In each case group (*a*) exhibited a superiority in survivorship which is far outside the limits of chance fluctuation. A comparison of mean survivorship of groups (*b*) and (*c*)

brings to light a difference of 0.767 ± 0.156 days ($4.91 \times$ P. E.), which difference is also a statistically significant one. In this case, group (c) possessed the greater survivorship value. These differences are illustrated by the survivorship curves in Fig. 4.

The influence of larval density on adult survivorship is clear, but the exact differential effect of the different densities is not so apparent.

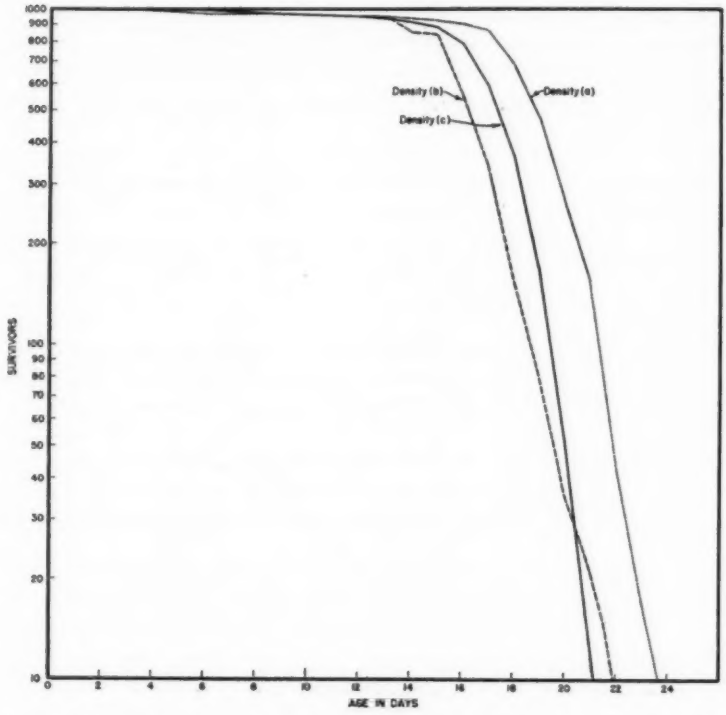

FIG. 4. Survivorship curves of starved adult *Tribolium* reared in various larval population densities.

It is true that group (a) which showed the most rapid larval development also exhibited the longest record of survivorship. But group (b), which showed the second most rapid larval development, survived the shortest period of time on starvation; and, conversely, group (c), which showed the slowest larval development, possessed the second best adult viability. These results indicate the desirability of further work to determine the differential effects on growth, development, and inherent

vitality of various sized total populations kept at a constant density per unit of environment.

This experiment has demonstrated that larval density is a factor which must be controlled in investigations on adult inherent vitality. From this it follows that the "capital" of the newly emerged adult *Tribolium* is not solely dependent upon the inborn organization of the organism, but also upon the environmental influences which have been effective throughout its immature stages. In a study such as that of Greiff (1940), where the length of life of isolated adults of *Drosophila melanogaster* and that of its mutant, Ebony, under conditions of complete starvation were compared, and where it was admitted that ". . . the ebony mutant fly was observed to do better under laboratory conditions than the wild-type fly" (i.e., it produced higher larval densities in the stock bottles from which the flies were isolated), it would obviously have been desirable to control the larval density factor.

TABLE V

Length of larval period for descendants of parents of indicated ages.

Age of parents	Range		Mean length of larval period (days)	Median length of larval period (days)	Standard deviation (days)	Coefficient of variation (per cent)	*N*
	Min. (days)	Max. (days)					
1 month	30.5	54.0	37.516±0.154	37.416±0.193	3.830±0.109	10.21±0.29	279
6 months	25.5	48.0	33.896±0.175	33.618±0.219	4.235±0.124	12.49±0.37	265

To separate the environmental factor of larval density from the inborn organization variable, and to test the relationship between age of parent and inherent vitality of offspring, further experiments were made.

Influence of Age of Parents on Vitality of Adult Offspring

Larval Development.—Eggs from two different stocks, one a month old, the other six months old, were collected and kept separate for hatching, whereupon 300 larvae from each group were placed in flour in half-pint milk bottles at a density of 100 larvae to 100 grams of food. Every factor in the experimental procedure was held constant except that of the age of the parent generations.

The data on larval development of these two groups of organisms descended from parents of different ages are set out in Table V.

The mean figures for length of larval period for the two groups reveal a difference of 3.620 ± 0.233 days ($15.53 \times$ P. E.). The progeny of the six-months-old parents developed at a rate significantly more

rapid than those of parents one month old. The frequency distribution curves in Fig. 5 illustrate this point clearly.

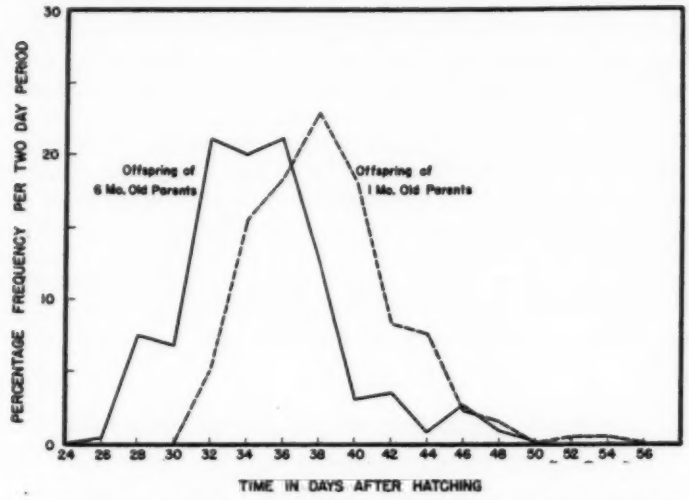
PERCENTAGE FREQUENCY PER TWO DAY PERIOD
30
20
10
0
Offspring of
6 Mo. Old Parents
Offspring of
1 Mo. Old Parents
24 26 28 30 32 34 36 38 40 42 44 46 48 50 52 54 56
TIME IN DAYS AFTER HATCHING

FIG. 5. Frequency distribution of length of larval period of beetles descended from stocks of various ages.

It is apparent that the six-months-old parents, having reached a more highly productive and more mature period of life, produced progeny which developed more rapidly than those produced by the younger, less productive, one-month-old parents. The variability in length of larval

TABLE VI

Length of life of starved adults descended from parents of indicated ages.

Age of parents	Range		Mean length of life (days)	Median length of life (days)	Standard deviation (days)	Coefficient of variation (per cent)	*N*
	Min. (days)	Max. (days)					
Male							
1 month	11.0	21.5	16.196±0.136	16.160±0.170	1.992±0.096	12.30±0.60	97
6 months	6.0	22.5	17.450±0.136	17.791±0.170	2.129±0.096	12.20±0.56	111
Female							
1 month	4.0	26.0	16.697±0.177	17.000±0.222	2.684±0.125	16.07±0.77	104
6 months	2.5	24.5	18.005±0.174	18.555±0.218	2.443±0.123	13.57±0.69	90
Total							
1 month	4.0	26.0	16.455±0.124	16.716±0.155	2.618±0.088	15.91±0.55	201
6 months	2.5	24.5	17.701±0.112	18.019±0.140	2.345±0.079	13.25±0.45	201

period for the two groups of progeny exhibited no striking difference. The larval mortality for the progeny of one-month- and six-months-old

parents respectively, was 3.67 ± 1.08 per cent and 11.67 ± 1.85 per cent. The difference here is 8.00 ± 2.14 per cent ($3.74 \times$ P. E.) and indicates a significantly greater amount of mortality in the progeny of the six-months-old parents than for those of one-months-old parents.

Survivorship of Adults.—Upon emergence, 201 adults from each group were subjected to complete starvation. The numerical data on

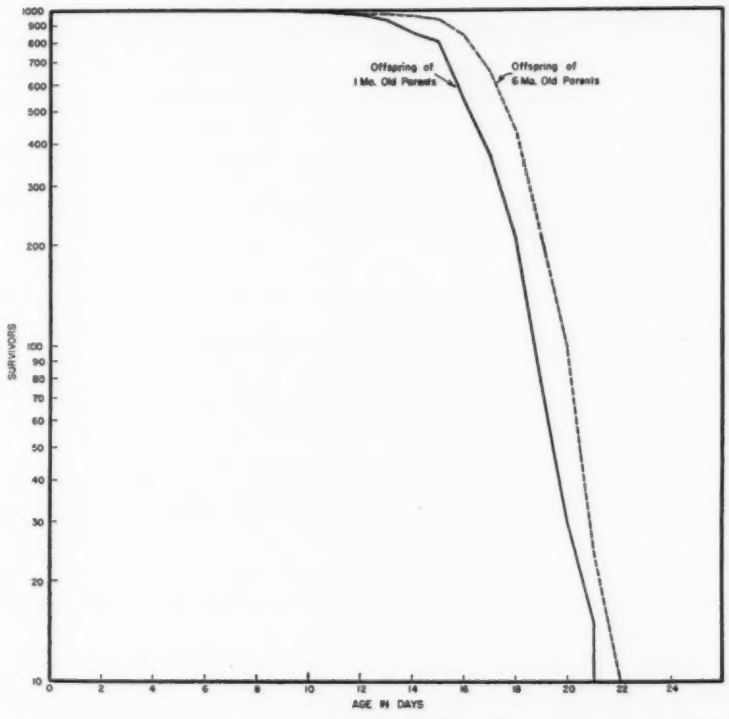

FIG. 6. Survivorship of starved adult beetles descended from parents of indicated ages. $N = 201$ in each group.

length of life of starved adults descended from parents one and six months old respectively are gathered into Table VI.

The sex difference in survivorship of the two groups is not large, though the females again showed a slight superiority. When the survivorship of the two groups is compared for either females, males, or for the combined sexes, there appear large differences. For the females, the difference is 1.408 ± 0.248 days ($5.68 \times$ P. E.); for the males it is

1.254 ± 0.192 days (6.52 × P. E.); and for the sexes together it is 1.246 ± 0.167 days (7.46 × P. E.). All of these differences are highly significant and in each case point to a longer survivorship for the progeny of 6-months-old parents than for those of one-month-old parents. The difference in survivorship of the two groups (sexes combined) is illustrated in Fig. 6.

The sex ratios of the two groups, though not significantly different from each other, or from a 50–50 ratio, are of interest in view of the fact that the parents which produced the most rapidly developing larvae and the most viable adults, also produced the highest proportion of male offspring. This observation is essentially in accord with that of Lawrence (1940) on *Drosophila melanogaster*.

Evidently parental age is a factor which must be controlled carefully in experiments on inherent vitality. It follows that parent age must be considered in determining the basic foundation of our knowledge of the nutritional requirements of *Tribolium*.

TABLE VII

Survivorship of adult Tribolium subjected, at the indicated ages, to a starvation diet.

Starvation begun	Range		Mean length of life after starvation (days)	Median length of life after starvation (days)	Standard deviation (days)	Coefficient of variation (per cent)	*N*
	Min. (days)	Max. (days)					
(*a*) At emergence...	4.0	26.0	16.455 ±0.124	16.716 ±0.155	2.618 ±0.088	15.91 ±0.55	201
(*b*) At age 20 days .	2.5	26.5	16.841 ±0.107	17.000 ±0.134	3.390 ±0.075	20.13 ±0.46	456
(*c*) At age 125 days	3.0	20.0	11.676 ±0.174	11.687 ±0.218	2.892 ±0.123	24.76 ±1.39	125
(*d*) At age 220 days	4.5	14.5	9.625 ±0.221	9.250 ±0.277	1.937 ±0.156	20.12 ±1.68	35

In order to determine the influence of the age of the adult beetle on its ability to survive conditions of complete starvation, a final experiment was performed.

Influence of Age on Inherent Vitality of Adults

Isolated adults fed a flour diet from the time of emergence to the time of starvation made up three of the four series of organisms in this experiment. Starvation was begun at ages 20 days, 125 days, and 220 days respectively in these three groups. Another group representing the controls were starved from the day of emergence. The beetles in all four series were descended from parents of equal age (six months) and were reared under identical larval densities (100 larvae in 100 grams of flour). The three groups of organisms that were fed for a while and then starved represent select groups, since only those remaining alive at

the desired time were used. The factor of selection does not impair the value of the experimental data, however, since we are not interested in the viability of those that died before a particular age, but only in the ability of those living at a certain age to survive when transferred to a starvation diet.

The data relative to the survivorship of these four groups of organisms are presented in Table VII.

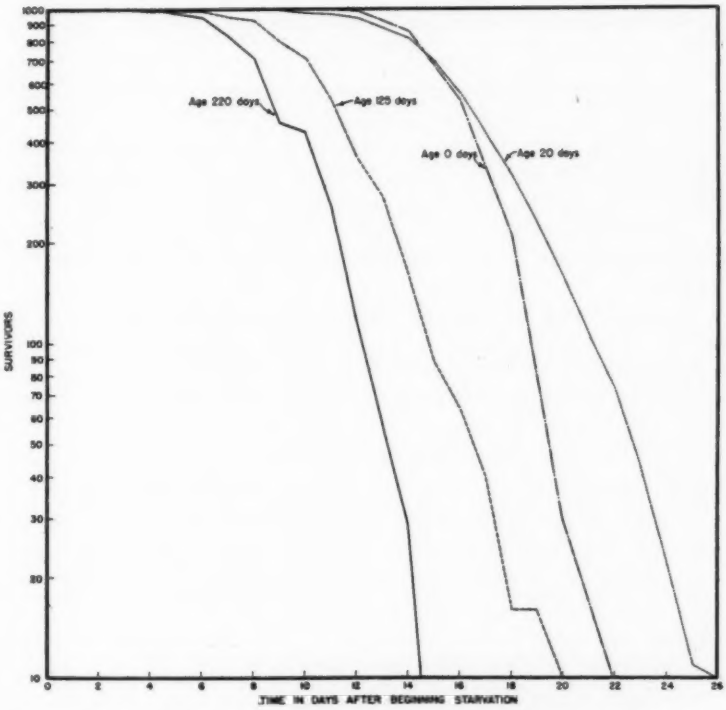

FIG. 7. Survivorship of adult *Tribolium* placed upon a starvation diet at various ages.

From Table VII it is evident that there exist significant differences between each two mean values except in the case of groups (*a*) and (*b*), which is only 0.396 ± 0.164 days ($2.41 \times$ P. E.). This difference, though statistically not certainly significant, points to a better survivorship of 20-day-old adults than newly emerged adults when both are subjected to starvation. The beetles of the two higher age groups all

survived a shorter period of time on starvation than did those of the two younger groups. The influence of age on the ability of adults to survive under conditions of starvation is illustrated graphically in Fig. 7.

The results suggest that the age at which the beetle survives best under starvation is something more than 0 and less than 100 days. The optimal point is probably in the neighborhood of 20 days. Investigations are now in progress to determine the exact age at which the longest survivorship under starvation occurs.

Summary

The experimental data set forth in this paper have served to show that:

1. Isolated adults of *Tribolium confusum* Duval, subjected to conditions of complete starvation upon emergence, or shortly thereafter, will survive up to 26.5 days depending upon the conditions of the experiment.

2. Survivorship of starved adults is shortened and the period of larval development is lengthened by an increase in larval population density.

3. Survivorship of starved adults is significantly longer and the period of larval development is significantly shorter for progeny of 6-months-old parents than for those of one-month-old parents.

4. Survivorship of starved adults is shortened with increasing age.

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THE PITUITARY REGULATION OF MELANOPHORES IN THE RATTLESNAKE

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Introduction

Only recently has the pituitary been linked with certain color changes in reptiles (Noble and Bradley, 1933; Kleinholz, 1935, 1938*a*, *b*; Parker, 1938). All of these observations are concerned with the lizard metachrosis and indicate that the pars intermedia of the pituitary gland plays an important rôle in the regulation of the melanophores. The present study reveals that a similar concept must be extended to the snake, since in this animal the dispersed phase of the melanophores is likewise dependent upon the pituitary secretion. Furthermore, certain aspects of the melanophores' arrangement and activity of the snake offer an interesting contrast to those described for the chromatophores of the lizard.

Material and Methods

This study deals primarily with the observations of the effect of hypophysectomies and subsequent replacement injections on the activity of the skin melanophores of the prairie rattlesnake, *Crotalus v. viridis* Raf. These specimens were collected in the eastern part of Wyoming. Similar experiments were later extended to several other species of snakes.

The operative removal of the snake pituitary is relatively simple, since the lower jaw can be retracted sufficiently to expose the entire roof of the oral cavity. Intraperitoneal injections of 10 per cent Nembutal served for anesthesia as recommended by Clark (1937). After the skin and muscles have been removed over the basi-sphenoid region the more exact site of the gland can usually be seen through the semi-transparent bone. The latter is chiseled away and then the whole gland may easily be removed. After this operation the bone capsule is replaced and the skin stitched. A strictly aseptic technique is not necessary.

The pars intermedia is very pale and may easily be distinguished from the highly vascular and pink pars anterior. Since the intermedia tissue in the rattlesnake is approximately one-third to one-fourth the size of the anterior lobe and rather loosely attached to the latter, it may be removed independently and completely.

The effect of intermedin (melanophore-dispersing hormone) on the operated animals was studied by means of intraperitoneal injections of an extract of the pars anterior of the chicken pituitary. This tissue contains an unusually high concentration of melanophore-dispersing hormone and was prepared and assayed according to the method of Kleinholz and Rahn (1939, 1940).

The effect of these various operations upon the ophidian melanophore could most easily be observed and studied in the living, anesthetized animal with a dissecting microscope, since the branches of these large chromatophores extend into the epidermal layer and are contrasted sharply with the homogeneous yellow background of the dermis. For permanent recordings of the various changes scales were clipped, fixed in alcohol and prepared as whole mounts. To supplement this series other scales were serially sectioned at 10 micra.

These operations yielded very striking results in the rattlesnake and led to similar experiments in other species of snakes. Although their gross color changes were not as striking, the melanophores responded in a similar manner to both hypophysectomies and injections. Altogether seven rattlesnakes were hypophysectomized and several of these animals were observed for four months after the operation. The other operated snakes included five garter snakes, *Thamnophis ordinoides;* one ribbon snake, *Thamnophis radix* (B. and G.); one bull snake, *Pituophis s. sayi* (Schlegel); and four Florida water snakes, *Natrix sipedon pictiventris* (Cope).

Observations

The background color of the prairie rattlesnake is a homogeneous light yellow color covered by various melanophores and pigmented epidermal cells whose groupings and concentrations form various light- and dark-brown scales. The latter are responsible for the typical color pattern of the rattlesnake (Fig. 2). Before describing the induced color changes, it seems desirable to consider first the histology of the rattlesnake skin, since the arrangement and activity of its melanophores do not correspond in all respects with those described for the lizards (von Geldern, 1921; Schmidt, 1917; et al.).

Epidermis

One of the outstanding features of this tissue is the occurrence of typical, branched melanophores and their processes which in the lizard seem to be confined almost entirely to the dermis. The epidermis is divided conveniently into two layers, the stratum germinativum and the stratum corneum.

The cells of the germinativum are cuboidal to squamous and arranged in two or three rows. Among these cells one finds the cell body and branches of a small melanophore which will be referred to as the epidermal melanophore (Figs. 1, 5), although the cell body may often lie just below the basement membrane. Whenever it appears in the epidermis, it is probably a migrant from below. One must recognize two varieties of this rather evenly branched epidermal melanophore. One is large (200 micra) and found chiefly on the dorsal side where it contributes to the color of the dark-brown scales (Fig. 5). The other epidermal melanophore is much smaller (130 micra) and confined chiefly to the lateral scales. This cell is more delicate in structure and contains relatively little melanin (Fig. 9).

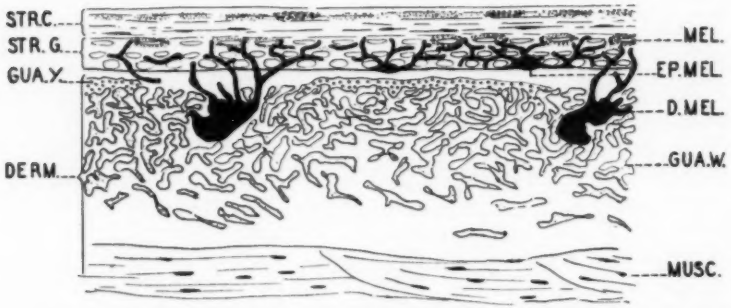


FIG. 1. Schematized section through a scale of the rattlesnake. The melanin deposits, *MEL.*, of the stratum corneum, *STR.C.*, are laid down first in the stratum germinativum, *STR.G.*, near the branches of the epidermal melanophore, *EP.MEL.*, and the dermal melanophore, *D.MEL.* The dermis, *DERM.*, contains a thick layer of white-reflecting guanophores, *GUA.W.*, covered by a thin layer of yellow-reflecting guanophores, *GUA.Y.* Muscle is denoted by *MUSC.*

The dermal melanophore (Figs. 1, 7) is discussed below. Yet, it must be briefly considered here as part of the epidermis, since its branches are found among the epidermal cells, while its cell body is always restricted to the dermis.

In the upper layer of the stratum germinativum the epidermal cells begin to keratinize and it is here that the first signs of intra and some inter-cellular melanin granules appear. Above this region is found the stratum corneum where the cells are completely keratinized and flattened. This region is destined to be cast at the next shedding. It has long been observed in older studies that this layer, the shedding skin, contains melanin granules which correspond in their horizontal distribution to the pigmented areas below. Whether or not these pigmented epidermal

cells (Figs. 1, 6) represent independent melanin producers is still questionable. At least they contribute greatly to the color intensity of the dark dorsal scales where they are especially prominent. However, they are not limited to this region alone, since they appear wherever melanophores occur and thus suggest a dependence on these cells.

Dermis

In this region are found the largest melanophores, the dermal melanophores (Figs. 1, 7) whose branches terminate among the epidermal cells. Their cell bodies lie imbedded among the dermal chromatophores responsible for the white-yellow background color of these animals. The latter pigment cells never extend into the epidermis. Their ramifying processes are filled with very small, alcohol-resistant crystals which reflect a white light and are doubly refractive under the polarizing microscope (*GUA.W.*, Fig. 1). Thus these cells may be regarded as guanophores according to the classification of Schmidt (1917). This white-reflecting guanophore stratum is in most places covered by a very thin layer of yellow-reflecting guanophores (*GUA.Y.*, Fig. 1) responsible for the almost homogeneous yellow background color exhibited by the scales. In a few places, this last-mentioned layer is absent (Fig. 1), such as the white-tipped dorsal scales and the regions directly above each dermal melanophore. In these places the dermis reflects only white color.

Effect of Hypophysectomy

The total removal of the pituitary as well as the extirpation of the pars intermedia alone causes a complete pigment concentration in all melanophore types. This would indicate that the pars intermedia is the only pituitary tissue responsible for the normally dispersed state of these cells. Evidence of a successful removal of this gland may be observed one hour post-operatively. Both types of melanophores begin to concentrate their pigment and the long branches of the dermal chromatophores give the impression of disappearing below the epidermis leaving small white islands in the otherwise yellow-reflecting guanophore area. After 4 to 24 hours the concentration is complete. The dermal melanophore pigment is clumped in an irregular fashion (Fig. 8). Granules of the guanophores do not seem to be affected.

It must be pointed out, however, that the snake as a whole does not necessarily appear lighter after the operation. This paling awaits the shedding of the stratum corneum, since this layer contains an abundance of already deposited melanin which may obscure any changes in the melanophores themselves. As soon as shedding has occurred, usually

three to four weeks after the operation, a striking change is observed (Fig. 2). (For similar observations on the induction of shedding in the snake after removal of the pituitary, see Schaefer (1933).) This paling is permanent, since little, if any, pigment is deposited hereafter.

Injection of Intermedin Hormone

The intraperitoneal injections of intermedin into an hypophysectomized animal cause complete dispersion in all melanophores. At least one hour is necessary before the first effects can be seen, yet 12 hours or more are required for the completion of this process (Figs. 4, 9). Large doses (several thousand *Anolis* units) will maintain complete dispersion for several days. The dermal melanophore dispersion, however, seems to be maintained by a lower intermedin concentration, since it may remain in this phase for several days after the epidermal melanophores have already completely concentrated their pigment.

It is interesting to observe that an hypophysectomized rattlesnake which has attained maximal pigment dispersion resulting from an intermedin injection is notably lighter than a normal animal (Figs. 3, 4). This emphasizes again that much of the color intensity of this animal is due to the melanin deposits in the keratinized portion of the epidermis and not entirely to the state of melanin dispersion in the chromatophores.

PLATE I

Explanation of Figures

2. Two normal (dark) and two hypophysectomized (light) prairie rattlesnakes. The control animal (*x*) has just shed and appears lighter than the other control animal which is about ready to cast its skin.

3. An hypophysectomized rattlesnake before the injection of intermedin hormone (anesthetized animal).

4. Same animal as in Fig. 3 photographed 24 hours after the injection of intermedin hormone. Notice that in spite of maximal pigment dispersion it is still lighter than the normal control animal in Fig. 2. (For explanation see text.)

Figs. 5–9. Photomicrographs taken from whole mounts of various scales. × 140.

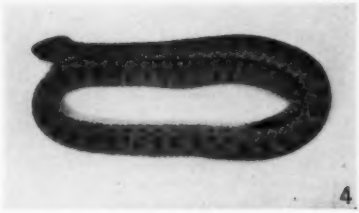
5. The large variety of epidermal melanophore which occurs primarily in the dark dorsal scales; maximal pigment dispersion.

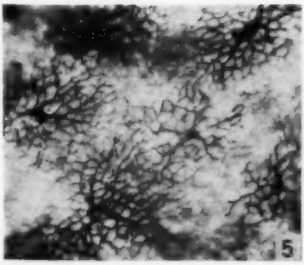
6. Melanin containing epidermal cells (*m.*) from a dark, dorsal scale surrounded by epidermal melanophores (*ep. m.*) whose pigment is concentrated.

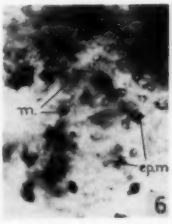
7. Dermal melanophores from a lateral scale, notice the bush-like appearance, finer branches, and lack of definite pattern.

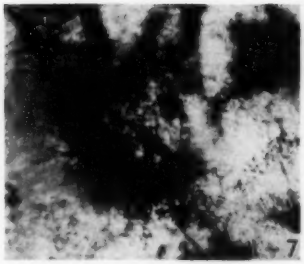
8. Lateral scale of an hypophysectomized animal showing pigment concentration in the small variety of epidermal melanophore (*ep. m.*) and in the dermal melanophore (*d. m.*).

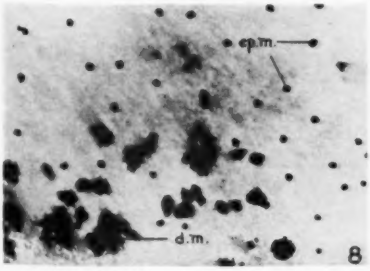
9. Lateral scale from same animal as Fig. 8; 6 hours after intermedin injection. Notice partial dispersion of pigment in both types of melanophores.











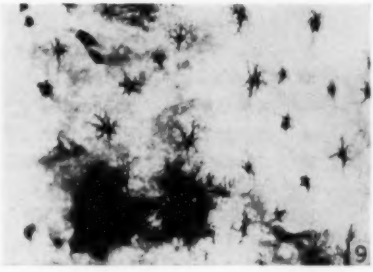


PLATE I

If intermedin injections were carried out for a considerable length of time in an hypophysectomized animal, one would expect to obtain the same dark color possessed by a normal animal due to the gradual deposition of epidermal melanin.

Other Snakes

A procedure similar to that outlined above was carried out with the other species of snakes mentioned. In all these forms suitable scales can be found which have small epidermal melanophores superimposed on a light dermis. These cells, however, account for very little, if any, color changes, yet will respond in the same way as the rattlesnake to hypophysectomy and intermedin injection. The color pattern of these snakes is accounted for primarily by a great concentration of various dermal chromatophores which have not been studied.

Discussion

The primary concern of this study is to point out that the pituitary and more specifically the pars intermedia of the rattlesnake is responsible for the dispersed phase of both types of melanophores. Noble and Bradley (1933) were first to associate the paling response of the lizard, *Hemidactylus,* with the removal of the pituitary. Similar observations were later made in much greater detail on *Anolis* by Kleinholz (1935, 1938*a, b*), and on the horned toad, *Phrynosoma,* by Parker (1938). Little seems to be known concerning the normal color changes in snakes. The experimentally induced metachrosis described above is relatively slow but definite, and this group of reptiles may now be included in the ever increasing number of vertebrates which have a pituitary regulation of the melanophores.

The occurrence of epidermal melanophores and dermal melanophores whose processes reach into the epithelial layer has long been recognized, but has received little attention (Kerbert, 1877; Krauss, 1906; Fuchs, 1914; Schmidt, 1917; Lange, 1931). They seem to be rare in the lizards (Schmidt, 1917) and are probably obscured in most snakes by the dark, dermal chromatophore layer. In the rattlesnake, however, these melanophores are especially striking, since their grouping and distribution seem to be responsible for the whole color pattern superimposed upon a uniform yellow, dermal guanophore layer. The melanophores are not crowded and are easily seen, since all their branches extend into the epidermal stratum. This species is consequently peculiarly adapted for this study. The other snakes studied are on the whole rather dark-colored forms and the activity of the epidermal melanophores can only

be recognized in a few light scales and even here they contribute little to the color pattern.

All authors who have studied the shed reptile skin, especially that of the snakes (Leydig, 1873; Werner, 1892; Lange, 1931) have observed the close coincidence between the melanin pattern of the shed skin and the pattern of the underlying layers. This can be followed especially well in the rattlesnake where the melanin deposits of the stratum corneum appear to coincide with the spread of each individual dermal and epidermal melanophore. Such a situation poses the still unanswered question of how this melanin is deposited. Is the melanin formed independently by the epidermal cells, as seems to be the case in various regions of the amphibian skin, or is it actually "injected" into the epidermal elements by the branches of the melanophores as Strong (1902) has described for the birds? In the snake the close association of all melanophore branches with pigmented epidermal cells suggests very strongly a mechanism of melanin deposition as in the bird feather. If this is actually the case, then one could expect complete cessation of all pigment deposits in an hypophysectomized animal, since the pigment is completely concentrated and would never reach the upper layers of the stratum germinativum where it seems to be normally laid down. Although all hypophysectomized animals remained permanently pale as long as four months after the operation, closer examination revealed slight melanin deposits in the epidermal cells. However, this does not necessarily invalidate this theory, since all these snakes received periodic intermedin injections and consequently might have had an opportunity to deposit pigment during these intervals of melanophore dispersion.

To what extent the color changes in the snakes can be compared with the relatively sudden changes described for the lizard group is difficult to state. In *Anolis* (von Geldern, 1921; Kleinholz, 1938*a*) the light (green) and the dark (brown) phases are due to the concentration and dispersion of pigment in the dermal melanophores. These melanophores never extend into the epidermis and achieve their effect by exposing or masking the green-reflecting chromatophores of the dermis. This metachrosis may be accomplished in a matter of minutes. In the rattlesnake the light and dark phases are accomplished experimentally in a similar manner, but the processes of the melanophores lie primarily in the epidermis, give rise themselves to definite patterns and are rather slow to react. As pointed out before, another factor must be considered in the light and dark phase of the snake. This is the heavy accumulation of melanin granules in the epidermal cells, which plays a minor or questionable rôle in *Anolis*. The melanin accumulation is especially noticeable in the darkening of snakes before they shed (Fig. 2), for at this

time the future stratum corneum has already formed under the old layer and undoubtedly contributes to the darkened condition as the new layer already carries considerable pigment. The amount of melanin deposits, however, seems to be in some way correlated with the melanophore activity, since hypophysectomized animals lay down very slight amounts of pigment, or no pigment at all, and remain permanently pale.

Exact studies concerning the effect of temperature and light on the chromatophore activity in snakes have not been found in the literature. The rôle of these two factors in the melanophore regulation of various lizards has recently received much attention by Kleinholz (1938*a*, *b*), Parker (1938), and Atsatt (1939), but whether or not snake melanophores will respond in a similar manner awaits further study.

Summary

1. In the prairie rattlesnake, *Crotalus v. viridis* Raf., the background of the skin is a homogeneous yellow-white color reflected from the evenly distributed dermal guanophores. The dark pattern of this snake is formed by various distributions of melanophores and pigmented epidermal cells superimposed upon this background.

2. Two main types of melanophores are found in the skin. One is relatively small and resides primarily in the epidermis; the other is much larger, structurally different, and retains its cell body in the dermis, but sends its ramifying processes into the epidermal layer.

3. Both of these melanophores appear to be associated with the deposition of melanin granules in the keratinizing portion of the epidermis, since (*a*) the distribution of its melanin deposits coincides with the pattern of the underlying melanophores, and (*b*) the rate of pigment deposition is greatly reduced after hypophysectomy.

4. The removal of the pituitary or the pars intermedia alone causes a permanent paling due to the complete concentration of the melanophore pigment. This paling, however, is more evident after the shedding of the old keratinized epidermal layer carrying previously deposited melanin.

5. Intraperitoneal injections of intermedin from the chicken pituitary will produce complete melanin dispersion in the melanophores of an hypophysectomized animal.

6. Preliminary observations on four other species of snakes indicate a similar pituitary regulation of the epidermal melanophores.

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DIPLOID AND ANDROGENETIC HAPLOID HYBRIDIZATION BETWEEN TWO FORMS OF RANA PIPIENS, SCHREBER[1]

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INTRODUCTION

One of the better methods for examining nucleo-cytoplasmic relationships is to combine identical nuclei with different cytoplasms. This can be achieved in a number of ways. For example, if the gametes of two different species are brought together to form reciprocal diploid hybrids, it is expected that at least in the early stages such hybrids will have identical nuclei and different cytoplasms. This arises from the fact that in most cases the maternal parent contributes practically all of the cytoplasm. Differences which may appear in the development and heredity of the reciprocals can therefore be related to differences in the egg cytoplasms of the two parent forms. If such cytoplasmic differences are observable and measurable, the possibilities are obvious.

The same end is achieved by combining the male nucleus of one species, subspecies or race with the nucleus-free egg cytoplasm of the same species and another species, subspecies or race. If the androgenetic or merogonic[2] homospermic haploid and heterospermic haploid resulting from this procedure show dissimilarities, these must be related to cytoplasmic differences.

It is scarcely necessary to point out that the results of such procedures seldom if ever satisfy the preconceived possibilities. The results from reciprocal diploid hybrids may be limited by an incompatibility of combined nuclei or nuclei and cytoplasms; or, where this is not the case, by the absence of sufficient cytoplasmic difference to produce an effect. And heterospermic haploids are usually less satisfactory. In the best of circumstances haploid organisms develop poorly and can only be produced in a limited group of materials. Apparently as a result of

[1] Data obtained, in part, from experiments performed during tenure of National Research Fellowship at Princeton University.

[2] The term androgenetic refers to the development of the *whole* egg with only the male nucleus functional; merogonic refers to the similar development of an egg-*fragment* (Wilson, 1925).

a high degree of incompatibility between nucleus and cytoplasm, the development of most androgenetic species hybrids is extremely abnormal and ceases in the earliest stages.

Despite such results, the possibility remains that hybridizations with heretofore untried material such as the North American Salientia may reveal one or more compatible combinations with the desired qualities. Experiments to test this possibility have been made and the following pages report one such investigation. Two distinct but closely related forms of the genus *Rana* have been combined reciprocally to form diploid and androgenetic haploid hybrids. The results form an interesting addition to the existing data on nucleo-cytoplasmic relationships.

The author is very grateful to Prof. G. Fankhauser for helpful suggestions and criticisms.

Materials

The gametes for these hybridization experiments were derived from two distinct forms of frog, one collected from the meadows of northern Vermont, the other from the immediate vicinity of Philadelphia. Both forms are commonly referred to as *Rana pipiens* and possibly represent different races or subspecies of that species. More attention will be given to their probable relationship in the discussion.

That the two forms are distinct is indicated by their general characteristics (Figs. 1 and 2), and also by the results of these experiments. These same features also indicate that the two are closely related. Therefore, as a temporary assumption and to facilitate the description of the experiments, the frogs are being considered as northern and southern forms or races of the same species. As such they will be referred to in the succeeding pages of this report.

A brief description, supplemented by Figs. 1 and 2, will indicate their major differences and similarities.

The northern form (from northern Vermont) is generally larger and, relative to its body size and weight, it has shorter jumping legs than the southern form. The head is obtuse; the vocal sacs on the male are less apparent; the dorso-lateral folds are broad; the skin is thick; the palmation is full. Distinctive features of pigmentation include spots that are larger and surrounded by a green or yellow border; the cross-bars on the tibia are generally complete; the posterior border of the thigh is marked by black spots on a continuous white background; the tympanum does not show a central light spot with the same clarity as in the southern form.

The southern form (from the vicinity of Philadelphia) is generally smaller and, relative to its body size and weight, its legs are longer. The head is more accumate; the vocal sacs are thin-walled and usually

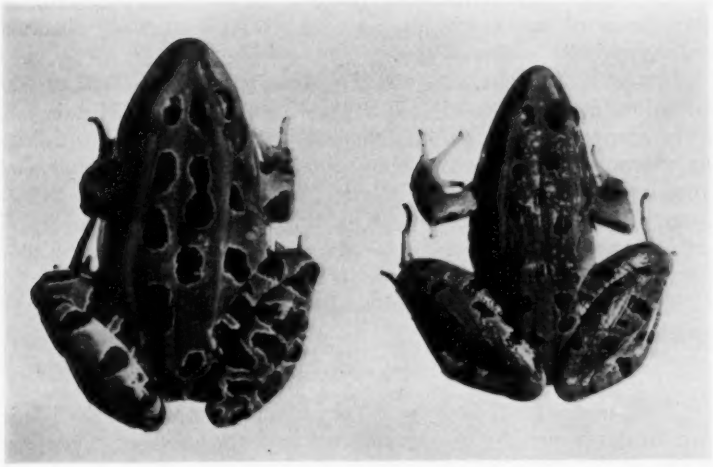

FIG. 1. Photographs of representatives of northern (left) and southern (right) forms of *R. pipiens* used in these experiments.

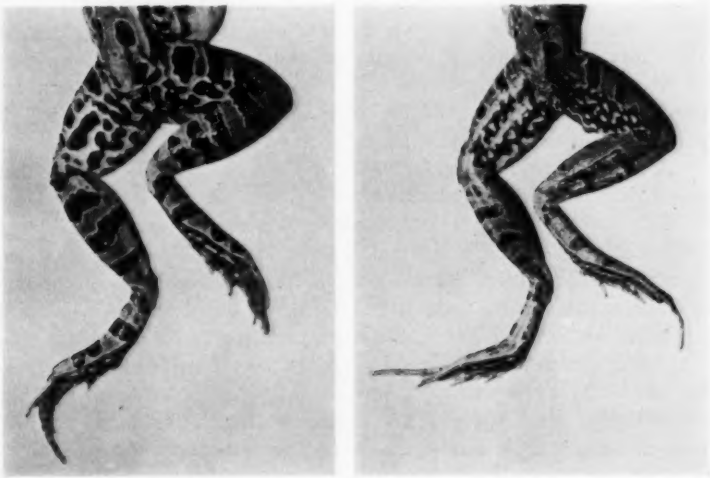

FIG. 2. Photographs showing pigmentation of jumping legs, northern (left) and southern (right).

apparent; the dorso-lateral folds are narrow; the skin is thin; the palmation is shallow. Distinctive features of pigmentation include spots that are smaller and, under the same laboratory conditions as the northern form, not surrounded by clear borders; the cross-bar markings on the tibia are generally interrupted along the dorso-lateral surface; the posterior surface of the thigh is marked by white spots on a continuous black background; and the tympanum generally shows a central light spot.

Methods

The eggs were obtained in every case from frogs which had been induced to ovulate by frog pituitary injections. As much care as possible was taken to avoid removing the eggs in the immature or over-ripe condition. The sperm for insemination were obtained by macerating the testes in 10 per cent Ringer's solution and every precaution was taken against contamination of one suspension with sperm from another.

The eggs of each frog were inseminated in two batches, the first with sperm of the same form, the second with sperm of the other form. Thus, in all, four batches of eggs were inseminated. An interval of 15 to 20 minutes was allowed to elapse between each insemination to provide time for removing the egg pronucleus from a number of eggs of each batch. By this procedure 8 different types of embryos were produced. These are listed below with the designation used for each in the balance of this report.

Homospermic diploids of the northern form	*n*
Homospermic haploids of the northern form	*n/2*
Heterospermic (hybrid) diploids from eggs of northern form and sperm of southern form	*ns*
Heterospermic (hybrid) haploids from cytoplasm of northern form and nucleus of southern form	(*n*)*s/2*
Homospermic diploids of the southern form	*s*
Homospermic haploids of the southern form	*s/2*
Heterospermic (hybrid) diploids from eggs of southern form and sperm of northern form	*sn*
Heterospermic (hybrid) haploids from cytoplasm of southern form and nucleus of northern form	(*s*)*n/2*

The egg pronucleus was removed with a fine glass needle as described in a previous report (Porter, 1939). Adequate numbers of pure and hybrid haploids were thus easily prepared (Table I).

All embryos were kept under identical conditions of temperature (19.4° C.) and space. In fixation of representative forms for a permanent record, a mercuric chloride, acetic acid, and formaldehyde mixture was generally used. The same sequence and time intervals were observed in fixation as had been observed in fertilization. Thus it was

assured that all animals fixed at the end of a period of time were of the same age.

Results

The description which follows is based upon observations made in the experiments listed in Table I. The possibility that the same results could occur by coincidence in all four series of crosses is slight if not negligible. The analysis is confined to such characteristics as were ap-

Table I [3]

Exp.	Date	Number of homospermic haploids produced	Number of heterospermic (hybrid) haploids produced	Treatment
1.	Jan. 9, 1939	23 *n/2* 21 *s/2*	41 (*n*)*s/2* 35 (*s*)*n/2*	Preliminary comparison of living animals made throughout development. Representative embryos fixed at end of 3, 5, 7, 9, 10 and 11 days.
2.	Jan. 17, 1939	29 *n/2* 37 *s/2*	30 (*n*)*s/2* 44 (*s*)*n/2*	Living animals compared throughout development. Representative forms fixed at end of 2, 3, 4, 5, 6, 7 and 8 days. Diploid hybrids and controls carried through metamorphosis for examination of inheritance.
3a.	Feb. 15, 1939	37 *n/2* 24 *s/2*	48 (*n*)*s/2* 43 (*s*)*n/2*	Living animals compared throughout development. Special attention given to gastrulation and neural tube formation. Representative forms fixed at end of 36, 43, 48, 51, 53, 55, 57, 59, and 61 hours and at 3 and 4 days.
3b.	Feb. 15, 1939	21 *n/2* 45 *s/2*	36 (*n*)*s/2* 41 (*s*)*n/2*	Living animals compared. Special attention given to study of older stages. Material fixed at end of 32, 54, and 60 hours and 3, 5, 6, 8, 9, and 10 days.

[3] The same females were used as a source of eggs for experiments 3*a* and 3*b*. Otherwise, different parents were used in each cross.

parent from external examination and only those characteristics which were uniformly shown by the animals in all four groups are stressed in the succeeding paragraphs.

For greater clarity the description of the 3-day-old embryos is presented first. With the differences of these in mind the descriptions of the younger and older stages have greater meaning.

Three-day-old Embryos

The following account is illustrated by the outline drawings in Fig. 3 and to them reference is constantly made.

The homospermic (control) diploids of the two races develop at approximately the same rate at 19.4° C. and, stage for stage, are comparable at the end of 72 hours. The differences, though real, are very slight and were clearly recognized only after repeated examination of material available. The northern diploids compared with the southern diploids show larger gill plates, a larger sense plate and larger mucous glands. Relative to body size the head of *n* is the larger. The neural tube is broader and stands up more distinctly in *n*. The tail-bud in *n* is smaller and directed more dorsally than in *s*, thus creating a deeper depression in the back of *n*. In relation to head size, the abdomen of *s* is larger than that of *n*. To these differences it can be added that the head flexure dorsal to the posterior margin of the gill plate is more pronounced in *s* than in *n*.

The homospermic (control) haploids of the two races, as is normal for haploids, are retarded in their development. Compared with each other they show in an exaggerated form the same differences that were given for the diploid controls.

The heterospermic (hybrid) diploids show approximately the same rate of development as the homospermic diploids and as each other. They differ in body proportions and show in accentuation the differences which are difficult to see between the pure diploids of the two forms. A greater proportion of *ns* consists of head structures than in the reciprocal hybrid. Conversely, a greater proportion of *sn* consists of abdomen and tail-bud. The mucous glands and sense plate are larger in *ns* and, posterior to the medulla, *ns* shows a smaller neural tube which terminates in a smaller and more dorsally directed tail-bud.[4]

The heterospermic (hybrid) haploids show in most exaggerated form the differences which have been referred to as existing between control diploids and haploids and more distinctly between the hybrid diploids. It is readily apparent that oral suckers, gill plates, and sense plate are greatly enlarged in *(n)s/2*. Relative to head size, the abdomen and tail-bud of *(s)n/2* are much larger than the same structures of *(n)s/2*. It can be further noted that the head of *(s)n/2* is flexed more ventrally than *(n)s/2* and the back of the latter is convex while in the former it

[4] If these experiments had been confined to the production and study of diploid hybrids, it is doubtful if the differences would have been considered great enough to warrant any conclusions. Supported by the evidence from androgenetic hybrids, however, the significance of the differences is unquestionable.

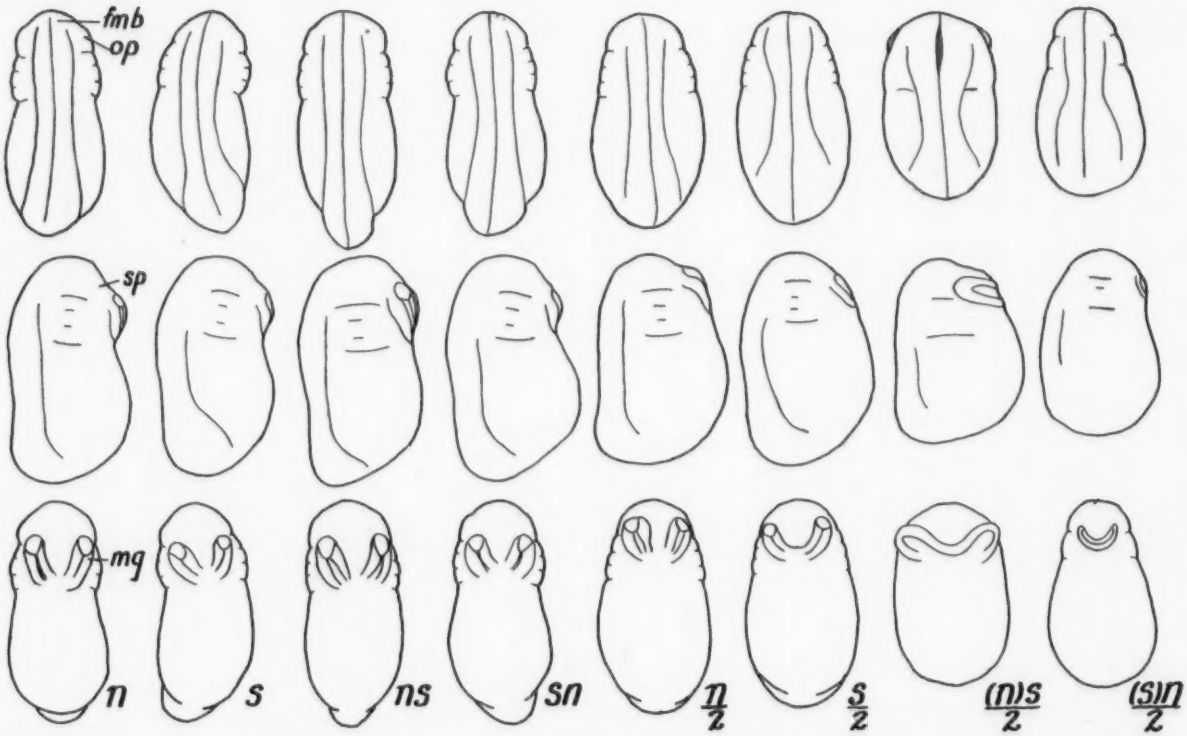

FIG. 3. Outline camera lucida drawings of dorsal, lateral and ventral views of 72-hour-old embryos. From left to right the drawings represent: *n*, homospermic diploid control of northern form; *s*, homospermic diploid control of southern form; *ns*, heterospermic diploid from northern egg and southern sperm; *sn*, heterospermic diploid from southern egg and northern sperm; *n/2*, homospermic haploid control of northern form; *s/2*, homospermic haploid control of southern form; (*n*)*s/2*, heterospermic haploid (androgenetic hybrid) from northern cytoplasm and southern sperm; (*s*)*n/2*, heterospermic haploid from southern cytoplasm and northern sperm. Magnification, 12.5 ×. The same sequence and magnification are observed in Figs. 4, 5, 6, and 7.

It can be seen that head structures (fore- and mid-brain regions, *fmb*, optic vesicle outpushings, *op*, sense plate, *sp*, and mucous gland, *mg*) are larger in *n*, *ns*, *n/2*, and (*n*)*s/2*, than *in s*, *sn*, *s/2*, and (*s*)*n/2* respectively. The tail-bud, *t*, tends to be larger in the latter. These differences are clear between the reciprocal heterospermic diploids, *ns* and *sn*, and pronounced between the heterospermic haploids, (*n*)*s/2* and (*s*)*n/2*.

is concave. These differences are the expression of the decidedly dissimilar embryology of the two reciprocal heterospermic haploids and not a difference in age or stage.

Summary.—In general the combinations which include cytoplasm of the northern form are characterized by larger head primordia and smaller posterior axial structures than are observed in those with southern cytoplasm. Such differences, only slightly apparent in the pure diploid controls, become progressively more accentuated in the homospermic haploids, the heterospermic diploids and in the heterospermic haploids.

It is of interest to observe now the earlier and later expressions of these general differences as shown by an examination of the earlier and later stages in the ontogeny of the various combinations.

Neural Tube Formation

The description under this heading is derived from a comparative study of living material and of representative embryos of the eight different types fixed at intervals of two hours from 51 to 61 hours after insemination. Illustration is provided by outline figures 4, 5, and 6 which are respectively representative of developmental stages reached at the end of 55, 59 and 61 hours.

The homospermic diploids, during this period, are very similar both in character and rate of development. As the neural plate is outlined, it becomes apparent that its anterior portion plus the sense plate are larger in *n* than in *s*. These differences increase in clarity as the neural folds are elevated and gill plates appear (Figs. 5 and 6). At this latter stage, *s* flattens dorsally and shows a greater elongation of that portion of the neural groove posterior to the gill plates. At the same time the neural plate and folds are more distinctly elevated in *s*. Although the neural plate and folds may be outlined in *s* slightly in advance of *n*, the closure of the folds is more rapid in the latter. During neurulation the blastopore of *s* is bounded laterally by distinctly thickened lips.

The homospermic haploids show in exaggerated form the slight differences existing between the diploid controls. In equivalent stages (55 hours) the neural folds of *n/2* are thicker, the sense plate and other primordial head structures are larger than in *s/2*, whereas the latter shows a greater elongation of the neural plate, especially that portion of it determined to be spinal cord. In *s/2* the neural folds show a greater elevation, the dorsal surface straightens or flattens out, and pronounced lateral lips bound the blastopore. Besides stage-for-stage structural differences, the differences in rates of separate morphogenetic processes are also accentuated. For example, it is noted that *s/2* completes gas-

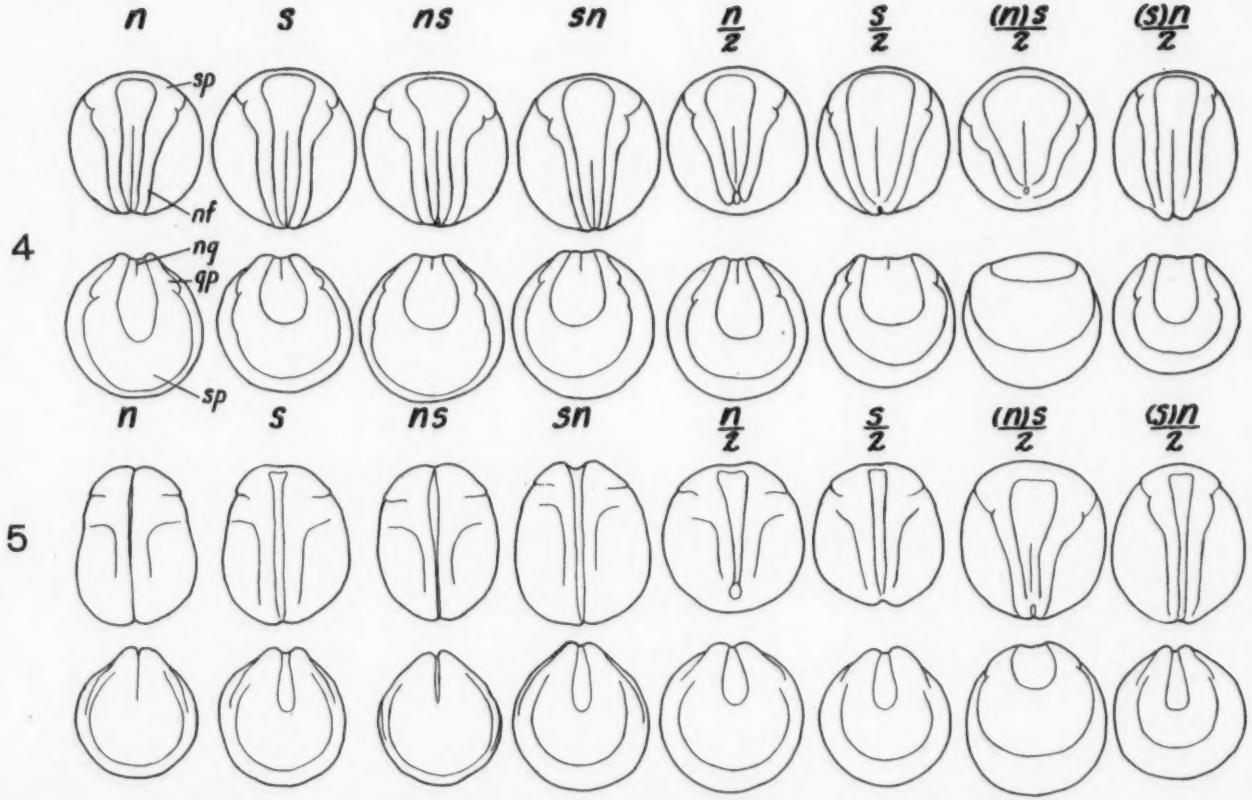

FIG. 4. Dorsal and anterior views of 55-hour-old embryos. It can be noted that, relative to size of abdomen, the head primordia (sense plate, *sp.*, gill plate, *gp.*) of *n*, *ns*, *n/2*, and *(n)s/2* are larger than the same of *s*, *sn*, *s/2* and *(s)n/2*. Neural folds, *nf*. Neural groove, *ng*.

FIG. 5. Dorsal and anterior views of 59-hour-old embryos.

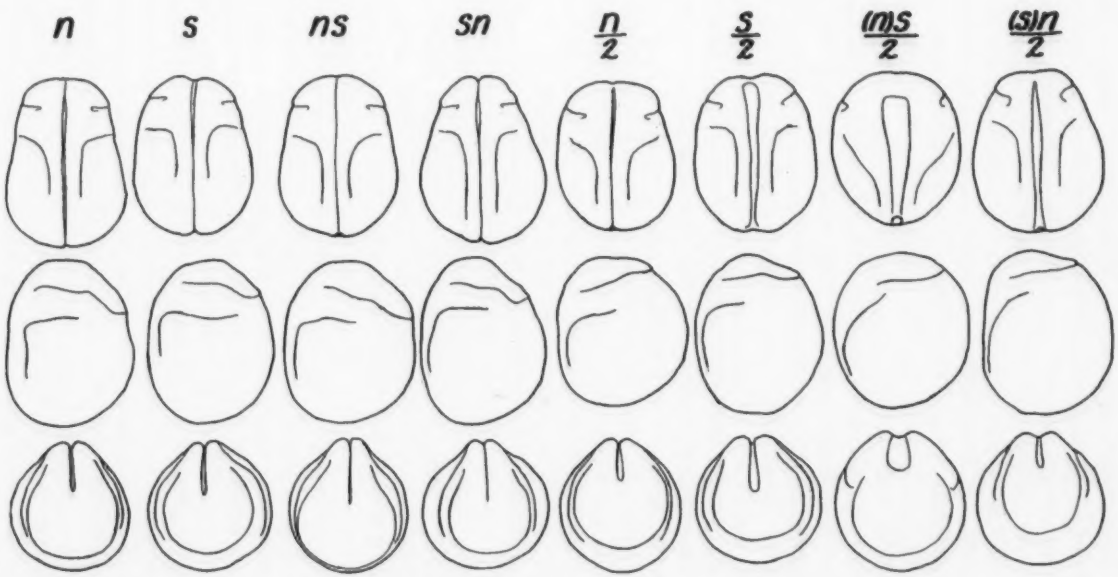

FIG. 6. Dorsal and anterior views of 61-hour-old embryos.

trulation considerably ahead of *n/2* and only slightly after *s*, whereas in *n/2* the neural folds appear to close slightly before they do in *s/2*.

The heterospermic diploids show clear-cut differences. Compared with each other in the early stages of neurulation, it is apparent that the *ns* forms have a shorter neural plate which is abnormally broad at the anterior end. The reciprocal *sn*, on the other hand, has a long and narrow neural plate. As the two differ from each other so do they differ from their maternal control diploids though to a lesser degree. Other features of dissimilarity include neural folds which are larger in *ns* than in *sn* and which are more distinctly elevated in *ns* than in the control diploid, *n*. In this latter respect they approximate the condition noted above as apparent in the paternal diploid control. As neural tube formation continues, the greater size of the head primordia and the shorter neural plate and groove are maintained in *ns*. Though the neural plate and folds are outlined almost simultaneously in these reciprocal hybrids, the folds come together in *ns* slightly before they do in *sn*. The lateral borders of the blastopore are swollen in *sn* to form lips as in *s* and *s/2*.

The heterospermic haploids show very striking differences during the development of the neural tube. At the end of 51 hours (not illustrated) *(s)n/2* has a dorsal flattened surface, abnormally straight from anterior to posterior ends. The neural plate is clearly outlined and is very narrow. Gastrulation has been completed, and there are extremely pronounced lips on both sides of the blastopore. Contrasting with this, the reciprocal *(n)s/2* is considerably retarded. The yolk plug is still apparent and the limits of the neural plate are not visible. The *(n)s/2* embryos are flattened dorso-ventrally and present a large, swollen appearance. By the end of 55 hours the neural plate of *(n)s/2* has been outlined. It is as broad as it is long and that portion designated to become neural tube is extremely short. The yolk plug persists. At this same time in *(s)n/2* the neural plate has lengthened and the neural folds have approximated to some extent. At 59 hours *(n)s/2* continues to show a short, broad neural plate, bounded by prominently elevated neural folds. This latter feature is a characteristic of *s* embryos and its appearance in these *(n)s/2* embryos represents the appearance of a specific paternal character. In the reciprocal it is not shown. It is a feature which will be easier of description and analysis when sectioned material is available. By 61 hours the neural folds of *(n)s/2* have started to approach and subsequent observations have shown that once started this process proceeds more rapidly here than in *(s)n/2*. At this time and later there is little elongation of the neural plate in *(n)s/2* and the yolk plug still persists in some cases. The sense plate and gill plates are abnormally large. The structure of the reciprocal hybrid *(s)n/2* at 61

hours is characterized by neural folds about ready to close, an elongate neural tube, and extremely small head primordia which foreshadow the diminutive head size of later stages.

Summary.—In summarizing, a few generalizations can be made. Those combinations which include cytoplasm of the northern race, including the diploid controls, are characterized by: (*a*) neural plates which when outlined tend to be shorter, and broader anteriorly, and (*b*) head primordia which are larger. The reciprocal combinations with cytoplasm of the southern race are, on the other hand, characterized by: (*a*) longer and narrower neural plates, (*b*) smaller head primordia, and (*c*) pronounced lateral lips on the blastopore. These differences become increasingly apparent as one compares respectively the diploid controls, the haploid controls, the reciprocal hybrid diploids, and the reciprocal androgenetic hybrids.

At one stage in the development of the neural folds it is apparent that they are more sharply delimited and distinctly elevated in the diploid of the southern form. This characteristic is repeated in the hybrid diploids and in the androgenetic hybrids containing the southern nucleus. It seems to represent, therefore, an inheritable embryonic characteristic capable of expressing itself in the foreign cytoplasm of the northern race. More careful analysis of this phenomenon is needed.

There are also to be noted slight differences in the times of occurrence and rates of the same morphogenetic processes. Relative to blastopore closure the neural plate is outlined earlier in those combinations with northern cytoplasm. Relative to time after fertilization, however, this may be later. Once clearly outlined the neural folds of the combinations with the northern cytoplasm seem to close more rapidly.

Gastrulation

This phase of the embryology of these various combinations was studied from living material and from representative forms fixed at the end of 36, 43, and 48 hours. A few differences between the gastrulae of those forms with northern cytoplasm and those with southern cytoplasm occur consistently (excepting the diploid controls where they are not sufficiently pronounced to be clearly evident) and become progressively more pronounced in haploid controls, heterospermic diploids, and heterospermic haploids. Those combinations with the cytoplasm of the southern race show a larger gastrular angle, a smaller completed blastopore, epiboly largely from the dorsal and lateral borders of the blastopore, and toward the end of gastrulation, an increasing thickening of the lateral blastopore lips. Those combinations with northern cytoplasm show a smaller gastrular angle, a larger blastopore, epiboly from all sides of

the blastopore, and thin blastopore lips. Gastrulation appears to begin earlier in *s*, *s/2*, *(n)s/2*, and simultaneously in *sn* and *ns*. Observations recorded on this feature and on the rate of gastrulation are not sufficiently extensive to be conclusive.

It would seem that the greater gastrular angle and the greater epiboly of the dorsal lip in those haploid and hybrid embryos with the southern cytoplasm are the early abnormalities related to the longer neural plate of later stages. It also appears that the thickened lateral blastopore lips of these same forms are the early expression of the larger tail-bud and somites of later stages (Bijtel, 1931). The opposites of these same features in those forms with the northern cytoplasm are probably related to the shorter neural plate and smaller tail-buds of their later stages.

Older Stages (4–10 Days)

The studies reported in this paper have been largely devoted to the younger stages hence only the most general features of the older stages will be described under this heading. Reference should be made to Fig. *7*.

The homospermic diploids of 4 and 5 days continue to show the slight differences which existed between the 3-day-old embryos. In the older stages, however, these differences become increasingly subtle. Relative to body proportions, the head of *n* remains larger while the tail of *s* is more elongate and larger in relation to the rest of the embryo. The dorsal concavity of *n* persists in greater prominence than in *s*.

The homospermic haploids differ in the older stages, as they had in the earlier stages, in relative size of body parts. The differences are similar to but more distinct than those occurring between the diploid controls.

The heterospermic diploids demonstrate more clearly the perpetuation of early differences. The combination, *ns*, persists in showing at various ages a larger head with larger mucous glands and a smaller dorsally directed tail. The converse of these features are shown by the reciprocal. Such differences are retained into the later stages of development, especially the relative head and tail size. Clear-cut appearance of paternal characteristics is recognized first in stages showing chromatophore patterns.

The heterospermic haploids, as in the younger stages, show the most striking differences. It is recognized, however, that these differences are less pronounced in the older stages suggesting some regulation. It is easily noted that the head of *(n)s/2* and its component structures remains larger and the tail remains smaller and directed dorsally. The androgenetic hybrid, *(s)n/2*, on the other hand, is characterized by a

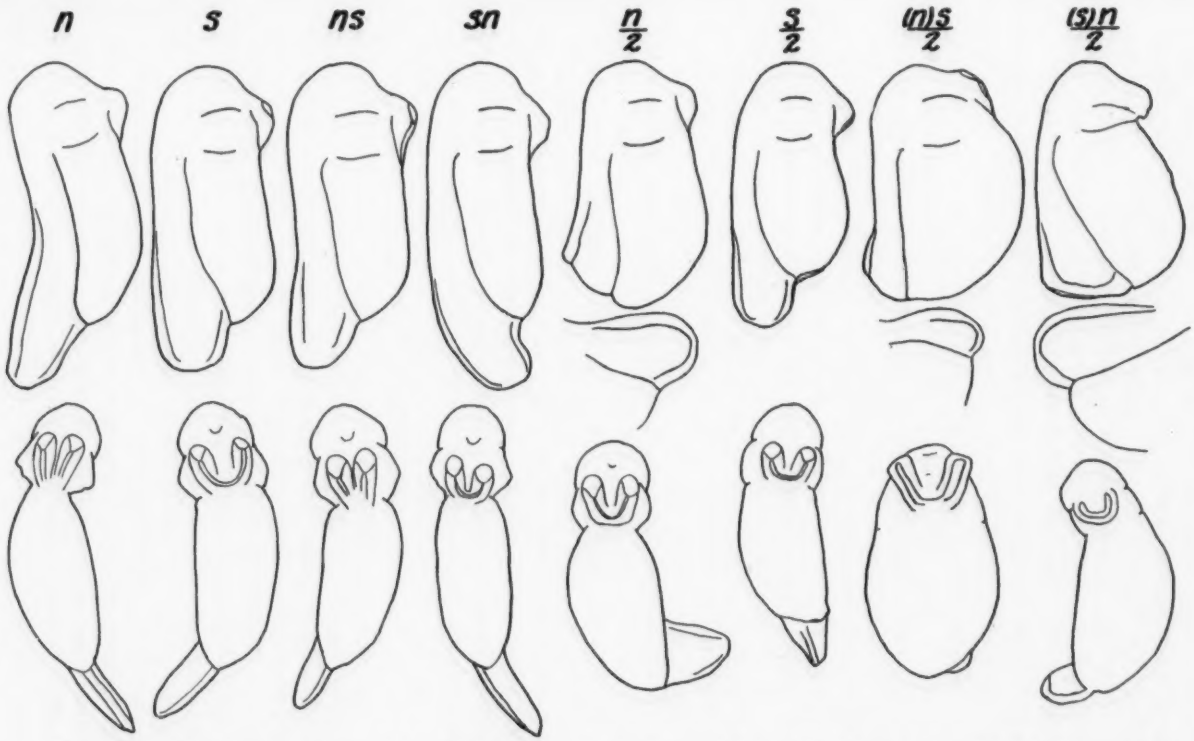

FIG. 7. Lateral and ventral views of 4-day-old embryos. It can be noted that early differences in relative body proportions persist in the various combinations.

small head and a ventrally directed tail which is referred to as larger because of the broad heavy somite mass at its base. These features were foreshadowed in the early embryonic development.

Summary.—Those combinations with cytoplasm of the southern race tend to have smaller head structures and, relative to body size, larger tails than the reciprocals with northern cytoplasm. Such features are doubtless the expression of earlier embryonic differences in the size of head primordia and tail-buds.

Survival of Various Combinations

Since representative embryos were sacrificed for fixation at various intervals, no definite data can be given to demonstrate survival value. Nevertheless, the observations made permit the following statements.

The heterospermic hybrids, in the majority of cases, develop up to and through metamorphosis. Beyond that stage no data are available.

The homospermic haploids of the two races demonstrate approximately the same viability. They continue their development, on the average, for from 8 to 12 days up to approximately stage 24 (Shumway, 1940).

The heterospermic haploids develop through the early stages showing only a small percentage of deaths. About one-fourth to one-third fail to hatch and of those which hatch the majority live for from 7 to 11 days or up to stages 22 and 23 (Shumway, 1940). They are slightly less viable than the haploid controls. In a small percentage of cases a gill circulation is established and in scattered cases growth continues sufficiently long to show the first guanophores. No differences in viability were recorded as existing between the two reciprocal combinations.

Identification of Haploids

The haploids were identified as such solely on the basis of the type of development. In an earlier study (Porter, 1939) it was shown that embryos which arise from operated eggs can be expected to develop as haploids in 90 per cent of the cases. Furthermore, such haploids were found to show certain definite characteristics when compared with their diploid controls. Hence, in these experiments, it has not been considered necessary to make a complete cytological examination of every embryo which developed from an operated egg, especially since group characteristics rather than individual characteristics have been considered. The isolated cases of diploidy which did appear among the embryos from operated eggs were readily identified by their development, cell size, etc.

In order to establish the chromosome count of the southern form,

the tail-tips of several haploids were examined. The examinations made indicated 13 to be the haploid count. This is the same as for the northern form determined in an earlier study.

Other Observations

In the case of two of the above-described experiments, surplus diploid embryos both pure and hybrid were kept for examination as older tadpoles and as metamorphosed frogs. The pure diploids showed differences characteristic of the northern and southern forms; the hybrid diploids showed blended inheritance with indications of stronger paternal influence in certain features of pigmentation. Thus despite the blending the reciprocal hybrids were distinguishable. These observations indicate that at least the differences in pigmentation between the two parent forms are related to differences in nuclear factors.

Two further observations, which, because of the small amount of evidence supporting them, must be considered as very preliminary, are briefly described. They are presented because of their interest as possible leads for experiments aimed at determining the nature of the factors responsible for the peculiar development of the hybrid embryos described above.

Since the frogs used were obtained from widely separated northern and southern points, it was considered of interest to examine the effect of high temperatures. Embryos representative of the 8 different combinations treated above were placed in a warming oven at 28°–29° C. Those combinations with cytoplasm of the southern form were not apparently damaged and developed in the typical manner, whereas those combinations with northern cytoplasm were markedly affected and only a very small percentage of the original number developed through 6 days. Controls kept at 19.4° C. developed normally.

Cytological examination of a few pure diploid 3-day-old embryos revealed some interesting differences in nuclear size and size of yolk granules. Measurements of nuclei of identical tissues of the two forms showed those of the northern to be the smaller. Measurements of the yolk granules revealed those of the northern to be much the larger.

Discussion

The discussion which follows will be confined to a consideration of the probable relationship of the animals used and to the more general aspects of the cytoplasmic and nuclear influences demonstrated. A detailed and inclusive treatment must await the accumulation of data from a more thorough study of these and similar hybrids. In a sense, then, this constitutes a preliminary report.

When the experiments were first undertaken the parents were thought to represent two distinct species. This conclusion was based on differences which the animals showed and also on the authority of amphibian taxonomists (Kauffeld, 1937; Stejneger and Barbour, 1939). An examination of the literature soon revealed, however, that considerable confusion exists in the classification of the leopard frogs or frogs of this type resident in the eastern states and possibly over a wider area. Differences between those forms resident in northeastern and those in the southeastern states have long been recognized, but it appears that sufficient material from a variety of localities has never been examined to make a conclusive analysis of the species. A brief reference to the writings of a few authorities on Salientia classification will serve to illustrate this confusion.

It should be recalled that the southern forms used in these experiments were collected in the vicinity of Philadelphia and the northern forms in northern Vermont. From its place of collection, the southern form doubtless coincides in appearance with that type early described by Schreber (1782) as *Rana pipiens* (Kauffeld 1936 and 1937). Later, Cope (1889), from examination of forms collected in a variety of localities, chose to describe the leopard frogs under three subspecies. The southernmost type he called *Rana virescens sphenocephala;* the type from the Atlantic coast *Rana virescens virescens* (probably same as Schreber's *R. pipiens* and the Philadelphia type of this study); and the type of northern distribution he called *Rana virescens brachycephala* (his description of which coincides perfectly with the northern form used in these experiments). More recent authors (Wright, 1933; Dickerson, 1906) have pictured and described the northern form as the typical *R. pipiens* and both it and the southern form have been considered as such by teachers and investigators alike. Most recently Cope's nomenclature has in part been revived, only instead of using a subspecies classification, the three types have been placed in separate species. Thus the most southern form is called *R. sphenocephala,* the Philadelphia form falls within the range of *R. pipiens* and the northern becomes *R. brachycephala* (Kauffeld, *loc. cit.,* and Stejneger and Barbour, *loc. cit.*).[5] It was on the basis of this latter classification that the frogs were originally considered to represent two species, *R. pipiens* and *R. brachycephala.* It is clear, however, that this classification is uncertain and consideration of some further points increases this uncertainty.

In the first place, it would seem that the two forms hybridize too

[5] In footnote, Stejneger and Barbour (1939) indicate that the whole sphenocephala-pipiens-brachycephala complex needs further examination and possible revision.

successfully to be representatives of two distinct species. It is true that a few distinct species of the Salientia have been successfully hybridized (Born, 1883; Pflüger and Smith, 1883; Heron-Royer, 1891; Montalenti, 1933; Durken, 1938 and Moore, 1940) so that the successful crossing of these two forms, even if they represent distinct species, is not without parallel. What is unique is the result of androgenetic hybridization, for no case involving the Salientia has been reported in which the development of an androgenetic or merogonic species hybrid continued to the advanced stages obtained in these experiments.[6] In other words, the compatibility of the two forms is greater than would be expected of two distinct species.

In the second place, it can be said that the characteristics of the two forms do not differ sufficiently to place them in separate species. Aside from body proportions, which is dealt with below, the major difference is one of pigmentation. This difference, it can be noted, does not involve the pattern but chiefly the size of the markings and intensity of the coloration. These are features which in other animals may vary considerably among races.

Finally, recalling that the two forms were collected from different northerly and southerly climates, and considering the points about to be discussed, the differences in body proportions likewise do not support a species relationship. Taxonomists have long been acquainted with certain generalizations known as the Bergmann and Allen rules pertaining to differences in size and body proportions which can be recognized between the northern and southern races of warm-blooded species. The former of these states that northern races are larger; the latter, that the southern races have relatively longer body projections. Within recent years an increasing volume of research examining racial and subspecies differences has shown that characteristics other than body size and proportions may likewise vary in an orderly and predictable manner with a variety of environmental gradients. Inclusive surveys of these phenomena are to be found in the recent writings of Goldschmidt (1940), Rensch (1936) and others. But among the species of animals examined for chains of racial differences or "clines" (Huxley, 1938), it appears that species of Amphibia have been regrettably absent. Schmidt (1938) reviewed some measurements of species of Salientia and noted that

[6] Baltzer (1920 and 1933) reports that from the combination of *Triton taeniatus* cytoplasm and *Triton palmatus* nucleus heterospermic haploids develop to stages showing good eye formation, pigment, small branching gills, and pulsating heart. Though this represents advanced development as compared with the usual result with different Salientia *species*, the stage reached does not seem to be the equivalent of that reached by the best of the heterospermic haploids obtained with these two *forms* of Rana pipiens.

relative to body size the leg length was greater for those representatives of a species which were collected from the more southern localities. The small number of animals examined and the preserved condition of these did not, however, permit any definite conclusions. Measurements of unselected groups of the two forms used in these crosses show the same tendency of the northern form to have a heavier and larger body structure relative to leg length.

It is possible that a thorough examination of the literature would reveal additional references to racial differences between frogs. For example, such differences are briefly mentioned in a paper by Pflüger and Smith (1883). Comparing the English race of *R. fusca* with the Königsburg race of the same species, they write:

> "Der englische braune Grasfrosch ist etwas kleiner und schlanker als der deutsche, weniger stumpfschnauzig und von zarterer Haut."

The similarity between these differences and those noted between the Vermont and southeastern Pennsylvania forms of *R. pipiens* is obvious. This similarity takes on added interest when it is noted that roughly the same climatic differences (as indicated by mean annual temperatures) exist between East Prussia (44° F.) and England (50° F.) as between Vermont (43° F.) and southeastern Pennsylvania (52° F.).

In view of these observations and the fact that racial variations accompanying climatic gradients have been found in a great many species of both the animal and plant kingdoms, it seems probable that species of frog when thoroughly examined will likewise show various clines with regard to temperature and other environmental factors. In the meantime it can only be maintained that the two forms used in these crosses probably represent two races of the same species.

If such is the case, the results of these experiments are of interest in demonstrating that racial differences involving body proportions can be recognized in early embryonic stages, and that at least some of the factors responsible for these differences exist in the cytoplasmic organization of the egg (see below). This observation and others which will probably be made from a more extensive examination of these and similar crosses may prove of interest to students who concern themselves with factors involved in species formation.

Experiments examining the relative rôles of the nucleus and cytoplasm in heredity have generally shown the nucleus to be the sole bearer of factors controlling the appearance of specific adult and juvenile characteristics. Some of these experiments have combined the nucleus of one species with the cytoplasm of another to form merogonic hybrids, attempting thus to demonstrate the presence of hereditary units in the

cytoplasm. Among these, the studies of the Hertwigs, Boveri, Baltzer, Hadorn, and Hörstadius are well known and frequently reviewed. With the possible exception of Hadorn's (1936) results, the demonstration of cytoplasmic inheritance has not been conclusive. The development of the merogonic hybrids generally ceases very early and even where it continues to a stage showing distinct species characteristics, as in certain sea-urchin merogons, the intermediate condition of the characteristic can be considered as an abnormality resulting from a degree of incompatibility between the nucleus and cytoplasm (Hörstadius, 1936). The early cessation of development which characterizes amphibian merogonic hybrids is probably also the result of a severe incompatibility.

It would appear that by using more closely related forms than those belonging to different species this problem of incompatibility could be overcome. To some extent this is probably true, but in using members of different races or subspecies, it is necessary to sacrifice the clear-cut distinctions which usually exist between the embryonic stages of different species and which are not to be expected between different races. Hence, the problem is fraught with difficulties and it is doubtful whether material such as used in these experiments, though it should be thoroughly examined, will supply any evidence in support of cytoplasmic-borne units of heredity even if present.

As distinct from heredity, cytoplasmic influence on development has been and can be demonstrated. This influence has been considered as the effect of plasmatic organization and composition upon the expression of nuclear factors. To this category of cytoplasmic activity the results of these crosses probably belong. Experimental embryologists have long recognized a high degree of cytoplasmic differentiation in a variety of eggs and the maintenance of such differentiation undisturbed is known to be essential in many cases for normal embryonic development. The cytoplasm of the egg by its organization, therefore, exerts an influence on the appearance of the adult in so far as this appearance is determined by the characteristics of the early developmental stages.[7] Needless to say, the nature of this early cytoplasmic influence is not understood but every new demonstration of its presence offers new possibilities for its examination.

The consideration of the results of these experiments is facilitated if the development of the two control diploids is visualized as paralleling on opposite sides an average or mean type (Fig. 8). If the factors responsible for this slight departure from the mean are nuclear and the

[7] In respect to even this cytoplasmic influence, it is to be remembered that considerable differentiation of the egg takes place in the presence of the maternal nucleus.

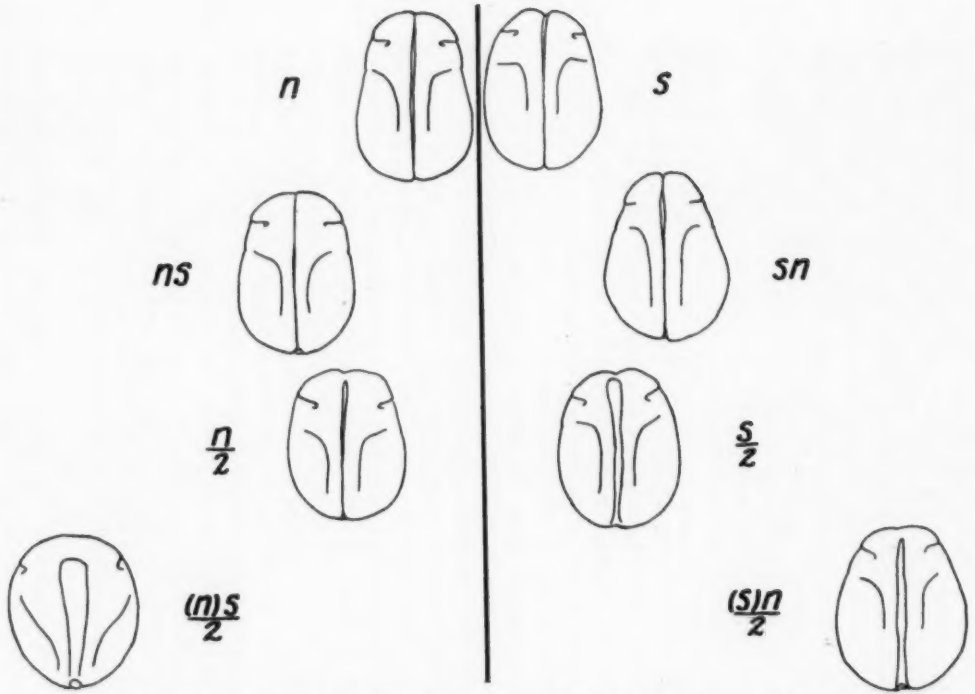

FIG. 8. Dorsal views of 61-hour-old embryos representing the development of the various nuclear-cytoplasmic combinations in relation to a hypothetical mean type, the central line. Those combinations with northern cytoplasm are on the left of the line; those with southern cytoplasm are on the right. The distance from the line represents the approximate degree of difference between the development of any one combination and the mean.

cytoplasms are perfectly neutral to nuclear control, then the diploid reciprocal hybrids would be expected to be identical and would in their development occupy a position coinciding with the hypothetical mean. Under the same conditions of nuclear control, the heterospermic haploid with the southern cytoplasm and northern nucleus would be expected to show the same development as the homospermic haploid of the northern form. Neither of these results is obtained. Instead, it is noted in the case of the reciprocal diploid hybrids that their development places them on opposite sides of the mean and at points more distant from the mean than their diploid controls. And in the case of the heterospermic haploids, the hybrid with the northern nucleus is not only further from the mean than the homospermic haploid of the northern form, but it is on the opposite side. Since the diploid hybrids can be considered as having identical nuclei and differing only in their cytoplasms, and since the same difference holds between the homo- and heterospermic haploids with nuclei of the same form, it follows that cytoplasmic influence is responsible for the dissimilarities existing between them.[8] Therefore, the eggs of the northern and southern forms differ in some property or properties of their cytoplasms.

Are the nuclei identical or do they also differ? If the nuclei are considered as being identical and responding solely to cytoplasmic influence, then the development of the reciprocal diploid hybrids should parallel the mean at the same distance as their respective diploid controls. Or, under the same assumed conditions of identical nuclei, the heterospermic haploid with the southern cytoplasm should be identical in appearance with the homospermic southern haploid. Again, the results indicate that the assumed condition of identical nuclei cannot be valid. On the other hand, the intermediate position of the diploid control between the mean and the hybrid diploid with the same cytoplasm indicates that the nucleus of each race has compensating factors for the cytoplasm of that race. The same conclusion is also supported by the intermediate position of the homospermic haploid relative to the mean and the heterospermic haploid with the same cytoplasm. Evidently then, the nuclei of the two forms also differ and do so in such a way as to compensate in development for cytoplasmic differences.

Cytoplasmic and nuclear differences seemingly demonstrated, it is of interest to determine which is responsible for the slight dissimilarities between the control diploids, and the more distinct dissimilarities between the homospermic (control) haploids of the two races. It has been shown that each diploid control in its morphogenesis is on the same

[8] A heterozygous genome in the parent forms could not account for these differences.

side of the mean as the hybrid diploid with the same cytoplasm, though not at the same distance. The homospermic haploids, in their development, parallel the mean at a greater distance than the control diploids, suggesting a lesser degree of compensation by the haploid nucleus. The homospermic haploid in its morphogenesis shows the same tendencies, though to a lesser degree, as the heterospermic haploid with the same cytoplasm. These facts suggest that the cytoplasmic differences are responsible for the slight dissimilarities between the diploid controls and homospermic haploid controls of the two forms. Further study may demonstrate whether or not these cytoplasmic differences are also related to the dissimilarities of the two adult parent forms.

What is the nature of these nuclear and cytoplasmic differences? There is not, of course, sufficient information available to answer this question. The presence of some degree of cytoplasmic organization in the amphibian egg has been shown to exist as early as 20 minutes after insemination (Fankhauser, 1930) and before first cleavage (Brachet, 1906), but the nature of this organization has not been demonstrated. Though the differences which are being examined cannot be described in precise terms, one feature of their relative nature does become apparent. It is clear from the results that some property or properties of the cytoplasm of the northern form tend to make the embryos with the cytoplasm of that form display certain features of development which, relative to the mean type representing normal development, are the exact opposite of those found in the embryos with the cytoplasm of the southern form (Table II). This infers that the differences in

TABLE II

Northern cytoplasm	Southern cytoplasm
1. *Small* gastrular angle	1. *Large* gastrular angle
2. *Large* completed blastopore	2. *Small* completed blastopore
3. Neural plate abnormally *broad* at anterior end	3. Neural plate abnormally *narrow* at anterior end
4. Neural plate abnormally *short*	4. Neural plate abnormally *long*
5. *Small* tail-bud	5. *Large* tail-bud
6. *Large* head primordia	6. *Small* head primordia
7. *Small* abdomen relative to head size	7. *Large* abdomen relative to head size

organization or composition, whether they be quantitative or qualitative, are of opposite natures as measured in terms of what they tend to produce in development. It has been noted further that the nuclei of the two forms have properties which tend to compensate for the cytoplasmic differences. Therefore the nuclei may also be considered to have properties of opposite nature. If this reasoning is correct, it seems that the nucleus of one form should supplement or enhance the cytoplasmic

influence of the other form. This means that the development of the reciprocal heterospermic haploids should be sufficiently different to suggest the activity of something more than the cytoplasm. While there is no unit of measurement by which the degree of difference can be determined, it is clearly great (Fig. 8) and is probably contributed to by a nuclear influence.

The differences in size of yolk granules and nuclei which preliminary studies have shown to exist between the early embryonic stages of the two forms constitute the only concrete dissimilarities between cytoplasm and nuclei so far observed. What connection, if any, these may have with the actual nuclear and cytoplasmic differences responsible for the above results is not readily apparent.

It is of further interest to determine how these differences operate to produce the results described above. This point is brought into this discussion not because any definite answer can be provided but because certain experimental treatments which could be expected to alter the mode of operation of cytoplasmic and nuclear factors have produced similar results. For example, if a temperature gradient is applied to the developing frog egg, that portion at the warm end of the gradient develops abnormally large structural units (Huxley, 1927; Dean, Shaw, Tazelaar, 1928; Gilchrist, 1928, 1929, 1933). More specifically, if the gradient is applied "adjuvantly" (Huxley, 1927) along the animal-vegetal polar axis in blastula stages (i.e., with warm end of gradient at animal pole) the tail-bud embryos from a blastula so treated have slightly larger heads than the controls and those subjected to the reverse gradient (Huxley and Dean, Shaw, Tazelaar, *loc. cit.*). It is further reported by the same authors that an adjuvant gradient increases by several times the normal difference in size existing between animal and vegetal cells of the blastula stages. Gilchrist (1933) demonstrates that size differences of embryonic structures resulting from temperature gradient treatments are not due solely to age differences but thinks rather that there is an alteration in what he terms the "physiological pattern" of the egg. In this same connection it can be noted that toxic agents applied to developing frog embryos can likewise produce a disproportion of parts most noticeably influencing those regions having the highest metabolic activity at the time of application (Bellamy, 1919).

With these results in mind, it is reasonable to suggest that the differences between the cytoplasms of the eggs of these two geographic forms or races are differences in factors which normally determine the varying rates of metabolism and cell division in the various parts of the developing blastula and possibly the induction processes in later stages. Only one bit of experimental evidence bearing on the physiological

properties of these eggs is available and this of a very preliminary sort—the temperature tolerance is higher for the egg of the southern form. This, it is logical to suppose, is related to the fact that the southern embryos may be called upon to develop at higher temperatures than the northern. From this, however, it is not possible to reason that other physiological differences which may exist between the two eggs are likewise related to climatic influences.

It is realized that other subjects of interest could be discussed in relation to the results of these experiments but it is felt that they may be considered more successfully after more information has been accumulated. For the present, it seems best to emphasize that the gametes of two geographic forms probably of the same species differ slightly in their cytoplasmic and nuclear properties and that by androgenetic haploid as well as diploid hybridization the orderly and measurable effects of these properties on early morphogenesis can be observed. The nature of these differences, their mode of operation, the relation of the embryonic differences they produce to the differences between the adults are among the major problems which can be and should be examined later with the same or similar materials and methods.

Summary

1. Two distinct forms of frog, commonly referred to as *Rana pipiens*, Schreber, are described, and evidence is presented to show that they probably represent geographic races of that species, one from northern Vermont, the other from southeastern Pennsylvania.

2. In the experiments described, the gametes of these two races have been combined reciprocally to form diploid and androgenetic haploid hybrids and the early development of these has been studied in detail.

3. The diploid hybrids developed through metamorphosis; the androgenetic hybrids for 7 to 11 days, up to about stage 24 (Shumway, 1940).

4. A comparison of 3-day-old control and hybrid embryos reveals that, in general, the combinations which include cytoplasm of the northern form are characterized by larger head primordia and smaller posterior axial structures than are observed in those with southern cytoplasm. Such dissimilarities, only slightly apparent between the homospermic diploid controls, become progressively more accentuated between the homospermic haploids, the heterospermic (hybrid) diploids, and the heterospermic (hybrid) haploids.

5. A study of gastrula, neurula, and older stages discloses the early expressions and later fate of the dissimilarities shown by the 3-day-old embryos.

6. These results demonstrate:

(*a*) Cytoplasmic differences between the eggs of the two forms which seem to have contrasting effects upon the same developmental processes.

(*b*) Nuclear differences which, in homospermic diploid control development, appear to compensate for the cytoplasmic differences.

(*c*) An orderly cytoplasmic influence on early morphogenesis.

7. The possible nature and mode of action of these differences are briefly discussed.

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THE RELATION BETWEEN HYDROGEN-ION CONCENTRATION AND VOLUME, GEL/SOL RATIO AND ACTION OF THE CONTRACTILE VACUOLE IN AMOEBA PROTEUS [1]

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INTRODUCTION

Von Limbeck (1894) observed that erythrocytes swell if the concentration of carbon dioxide in the blood is increased. Jacobs and Parpart (1931) found that hemolysis in erythrocytes increases with increase in acidity and that the effect of as small a change as 0.01 pH is measurable. Lucké and McCutcheon (1926) maintain that the volume of eggs of Arbacia in sea water does not change with changes in hydrogen-ion concentration between pH 4.2 and pH 9.8 unless the eggs are left so long that they become injured.

Chalkley (1929), in observations on *Amoeba proteus* in balanced salt solution, found that as the hydrogen-ion concentration decreases from pH 6 the volume decreases to a minimum at pH 7 and then increases, i.e. that there are two maxima, one in the acid range and another in the alkaline; and Mast and Prosser (1932) found that as it decreases over the range studied (pH 5.4–8) the gel/sol ratio decreases. Pitts and Mast (1934) investigated the relation between gel/sol ratio and hydrogen-ion concentration in single as well as balanced salt solutions. They confirmed the results obtained by Mast and Prosser and conclude that "in sodium or potassium salt solutions the gel/sol ratio decreases as the hydrogen-ion concentration decreases, but that in calcium salt solutions it increases in the more acid range (pH 5.0 to pH 5.9) then remains constant or decreases slightly."

Thus it will be seen that there is considerable difference of opinion concerning the effect of changes in hydrogen-ion concentration of the medium on the volume of cells, that no observations have been made on *Amoeba* concerning the relation between volume and hydrogen-ion con-

[1] These investigations were carried out under the direction of Professor S. O. Mast in the Zoölogical Laboratory of the Johns Hopkins University and the Marine Biological Laboratory at Woods Hole, Mass. They were greatly facilitated by a grant from the Brooks Fund.

centration in single salt solutions, and that the results obtained in observations on the gel/sol ratio in these solutions have not been confirmed. It is the purpose of this paper to present detailed information concerning the relation between hydrogen-ion concentration, volume and the gel/sol ratio of *Amoeba proteus* in salt solutions containing respectively sodium, potassium and calcium as the only metallic cations.

Material and Methods

The amoebae used were selected, prepared, and measured for volume and gel/sol ratio as described in the section on material and methods in a previous paper (Mast and Fowler, 1935). The solutions used consisted of a primary phosphate hydroxide buffer system in which the concentration of the cation was identical in the phosphate and in the hydroxide (Pitts and Mast, 1933). The stock solution of phosphate was kept in a covered Pyrex flask and the stock solution of hydroxide in a carefully sealed Pyrex flask open to the exterior through a soda lime tube and through a 50 cc. Pyrex glass buret. These solutions were standardized according to the method described by Pitts and Mast (1933). By mixing the phosphate and the hydroxide in various proportions the desired hydrogen-ion concentration was easily obtained. The hydrogen-ion concentration of each solution prepared was measured with a quinhydrone electrode and a Leeds Northrup potentiometer.

Volume and Gel/Sol Ratio

Sodium Salts

Ten amoebae were selected, put into modified Ringer solutions,[2] and left for approximately 24 hours. Then the volume and the gel/sol ratio of each were measured as described above, after which they were transferred to a solution containing 0.002 M sodium as the only metallic cation at pH 5.5, left 15 minutes and measured again, after which they were measured at 15-minute intervals for 105 minutes. This was repeated for 60 other individuals, 10 in each of the following solutions: 0.002 M sodium phosphate buffer solutions at pH 6.0, pH 6.5, pH 7, pH 7.5, pH 8.0 and pH 8.8, respectively. There was but little change in either the volume or the gel/sol ratio of the amoebae after they had been in these solutions 30 minutes. All the results obtained in the

[2] 3.3 cc. salt solution (0.35 gram NaCl, 0.14 gram KCl, 0.12 gram $CaCl_2$, 1000 cc. H_2O) + 5 cc. buffer solution (25 cc. 0.2 M KH_2PO_4, 12.5 cc. 0.2 M NaOH, 62.5 cc. H_2O; Clark, 1927) + 91.7 cc. H_2O. This solution is the same in composition as Chalkley's 1/60 Ringer solution (1929). The total concentration of salts is 0.002 M and the hydrogen-ion concentration is pH 6.8.

measurements of volume and gel/sol ratio made at each hydrogen-ion concentration were therefore respectively thrown together and the average calculated. These averages are presented in Fig. 1, *A*.

Figure 1, *A* shows that after the amoebae had been transferred from modified Ringer solution 0.002 M, pH 6.8, to 0.002 M sodium phos-

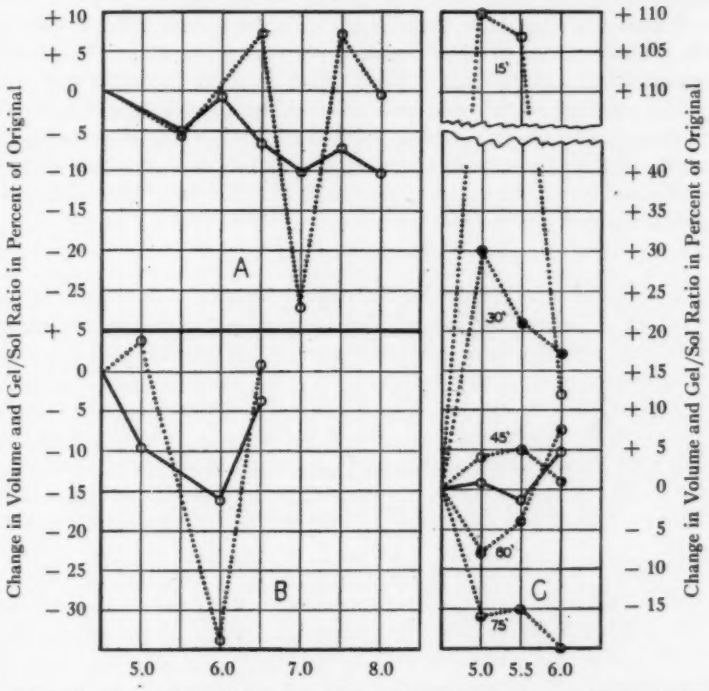

FIG. 1. The relation between volume and gel/sol ratio in *Amoeba proteus* and hydrogen-ion concentration in solutions which contain only one kind of metallic cations. *A*, sodium 0.002 M; *B*, potassium 0.002 M; *C*, calcium 0.005 M; solid curves, volume; broken curves, gel/sol ratio; 0, volume and gel/sol ratio in modified Ringer solution; +, increase in volume and gel/sol ratio; —, decrease in volume and gel/sol ratio; points on curves, averages of 3 to 50 measurements (see text); 15′, 15 min. after transfer from Ringer solution to Ca solution; 30′, 30 min.; 45′, 45 min.; 60′, 60 min.; 75′, 75 min.

phate buffer solutions at the various hydrogen-ion concentrations used, the average volume calculated from measurements made at 15-minute intervals for 105 minutes after transfer, decreased 5 per cent at pH 5.5, 0.3 per cent at pH 6.0, 7 per cent at pH 6.5, 10 per cent at pH 7.0, 7.3 per cent at pH 7.5 and 10 per cent at pH 8.0. (Measurements at

pH 8.8 were impossible because the amoebae disintegrated within a few minutes after they had been put into the solutions.)

This indicates that as the hydrogen-ion concentration in a sodium solution decreases from pH 5.5 the volume increases to a maximum at pH 6, then decreases to a minimum at pH 7, then increases to a secondary maximum at pH 7.5, and then decreases again.

Figure 1, *A* also shows that the gel/sol ratio decreased 5.5 per cent at pH 5.5, 27 per cent at pH 7 and zero per cent at pH 8, and that it increased 7.5 per cent at pH 6.5 and pH 7.5. This indicates that in a sodium solution as the hydrogen-ion concentration decreases from pH 5.5 the gel/sol ratio increases to a maximum at pH 6.5, then decreases rapidly and very greatly to a minimum at pH 7, then increases equally rapidly and greatly to a second maximum at pH 7.5 and then decreases again.

Potassium Salts

The experiments concerning the relation between volume and gel/sol ratio and hydrogen-ion concentration in solutions containing potassium as the only metallic cation were performed the same as those containing only sodium, except that three specimens were used for each hydrogen-ion concentration in place of ten. The results obtained show that there was, as in the sodium solutions, but little change in volume and gel/sol ratio after the amoebae had been in the solutions 15 minutes. The averages of all the results obtained concerning volume and gel/sol ratio were therefore respectively calculated. These averages are presented in Fig. 1, *B*.

Figure 1, *B* shows that in amoebae transferred from modified Ringer solution, pH 6.8, to potassium phosphate buffer solutions at various hydrogen-ion concentrations, the volume decreased 10 per cent at pH 5.0, 16.1 at pH 6.0, and 3.7 at pH 6.5, and that the gel/sol ratio increased 4 per cent at pH 5 and about 1 at pH 6.5 and decreased 33.8 per cent at pH 6. In neutral and alkaline solutions the amoebae disintegrated so rapidly that it was impossible to measure them.

These results indicate that as the hydrogen-ion concentration in potassium solutions decreases from pH 5 the volume of *Amoeba proteus* decreases slowly to a minimum at pH 6 and then increases rapidly; and that the gel/sol ratio decreases very rapidly to a minimum at pH 6 and then increases equally rapidly.

Calcium Salts

The methods used in the observations on amoebae in solutions in which calcium was the only metallic cation present are the same as those

used in the preceding experiments. The concentration of calcium was 0.005 M and ten amoebae were measured at each of four hydrogen-ion concentrations: pH 5, 5.5, and 6. In lower hydrogen-ion concentrations, it was impossible to maintain the concentrations long enough to make the measurements.

The results obtained show that there was but little change in volume after the amoebae had been in the solutions 15 minutes, but that the gel/sol ratio changes radically with time. In reference to volume, the average for all the measurements made at each hydrogen-ion concentration was therefore calculated; but in reference to gel/sol ratio the average of the results obtained in the measurements made after each 15-minute period at each hydrogen-ion concentration was calculated. These averages are presented in Fig. 1, *C*.

Figure 1, *C* shows that in the amoebae which were transferred from modified Ringer solution pH 6.8 to calcium phosphate buffer solutions the volume increased 0.8 per cent at pH 5 and 4.8 per cent at pH 6 and that it decreased 1.4 per cent at pH 5.5. This indicates that as the hydrogen-ion in calcium solutions decreases from pH 5 the volume decreases slightly to a minimum at pH 5.5 and then increases fairly rapidly.

This figure shows that during the first 15 minutes after the amoebae had been transferred from modified Ringer solution the gel/sol ratio increased 110 per cent at pH 5.5, 107 per cent at pH 6.5 and 12 per cent at pH 6, and that it then decreased during the following 90 minutes to 16 per cent below the original ratio at pH 5, 15 per cent at pH 5.5 and 20 per cent at pH 6.

This indicates that after transfer from Ringer solution to calcium solution the gel/sol ratio increases very rapidly and very extensively, if the hydrogen-ion concentration is relatively high, and then gradually decreases and that the extent of the change in this ratio varies directly with the hydrogen-ion concentration.

The Action of the Contractile Vacuole

The results presented by Chalkley (1929) and those presented in the preceding pages show that the volume of *Amoeba proteus* is correlated with the hydrogen-ion concentration of the surrounding medium. The question now arises as to whether or not this correlation is dependent upon the action of the contractile vacuole. This problem was investigated as follows.

Fifty to one hundred amoebae were transferred successively through three beakers each containing 50 cc. redistilled water and left in the last for one hour. During this time many of the amoebae became radiate in

form. About 25 of these were selected and put into 50 cc. 0.002 M Ringer solution (pH 6.8) and left 12–15 hours, then an actively moving specimen was selected and measured in the volumescope as previously described (Mast and Fowler, 1935). It was then transferred to test solution, in the depression on a Pyrex glass slide, and covered with a cover-glass, after which the diameter of the vacuole, immediately preceding contraction, and the interval between successive contractions were measured with a Filar micrometer ocular and with a stop watch respectively. This was continued as long as desired, after which the whole process was repeated with other individuals in this and in other solutions. Then the average volume of fluid eliminated by the contractile vacuole per amoeba per minute and the average volume eliminated per minute in percentage of the volume of the amoebae were calculated for each solution used.

There was considerable variation in given amoebae during the period of observation in the different solutions used and in the intervals between successive contractions, but these variations were not specifically correlated with time in any of the solutions except the Ringer-lactose solution, a solution in which the osmotic concentration was relatively very high. In this solution the size of the vacuole decreased and the interval between contractions increased with time and there usually were not more than five contractions before it ceased to function altogether. These statements are substantiated by the following typical results.

In one of the amoebae transferred to Na buffer solution (pH 6.5), the diameter of the first vacuole in the series of ten measured was 2.36 μ, that of the last 2.30 μ, that of the smallest 2.07 μ and that of the largest 2.66 μ.

In one of the amoebae transferred to the Ringer-lactose solution, the diameter of the first vacuole was 2.42 μ and those of the following three were 2.79 μ, 2.69 μ and 2.38 μ respectively, and the intervals between the successive contractions in the series were 2′ 45″, 3′ 5″, 5′ 0″ and 6′ 30″. After this series of contractions was complete the vacuole was continuously observed for 45 minutes. It did not contract during this time but it became smaller, the diameter at the end of three successive 15-minute intervals having been 1.75 μ, 1.66 μ and 1.57 μ respectively. In this amoeba the vacuole ceased contracting 17 minutes and 20 seconds after transfer to the Ringer-lactose solution. The average time required for cessation of contraction in this solution was 18.5 minutes.

The averages of the results obtained directly by observation of amoebae in the different solutions used and those obtained by calculations are presented in Table I.

This table shows that the size of the contractile vacuoles in the amoebae in all the different solutions used except the Ringer-lactose solution and the Na buffer (pH 8) was essentially the same.

In the Ringer-lactose solution the vacuole decreased markedly with time, as stated above, hence the low average diameter of 2.09 μ. The

TABLE I

The volume of fluid eliminated by the contractile vacuole in Amoeba proteus *in various solutions.* Temperature, 25° C. Ringer and Na buffer solutions are described in the text. The volume of only three of the nine amoebae in pure water was measured. The average volume for these is 4980 cμ and the average elimination 0.30 per cent of this volume.

Solutions used	Amoebae studied		Contractile vacuoles				
	Number	Av. vol. in 1000 cμ	Number measured	Av. diam. in μ	Av. interval between contractions	Av. vol. of fluid eliminated in cμ per min. per amoeba	Av. percentage of vol. of amoebae eliminated per min.
Ringer 0.002 M pH 6.8	10	1785	100	2.46	3′ 49″	5670	0.31
Ringer 0.002 M pH 6.8 −0.2 M lactose	10	?	42	2.09	9′ 47″	1500	0.008
Na buffer 0.002 M pH 5	5	1753	50	2.29	4′ 5″	4260	0.24
Na buffer 0.002 M pH 6	6	1891	60	2.25	4′ 10″	4620	0.24
Na buffer 0.002 M pH 6.5	6	1619	60	2.22	4′ 4″	3900	0.24
Na buffer 0.002 M pH 7	5	2184	50	2.43	5′ 31″	7380	0.17
Na buffer 0.002 M pH 8	10	3141	100	2.76	4′ 2″	7620	0.24
Pure water	9	1625	90	2.61	3′ 36″	4980	0.30

high average diameter of 2.76 μ in the Na buffer (pH 8) appears to have been directly correlated with the size of the amoebae in this solution.

The table shows that the average interval between successive contractions was relatively small in pure water and Ringer solution and considerably higher but essentially the same in all the Na buffer solutions

except pH 7 in which it was relatively very high. It shows that the rate of elimination per unit volume of protoplasm was relatively high in pure water and Ringer solution and considerably lower but essentially the same in all the Na buffer solutions except pH 7, in which it was much lower.

The results obtained consequently indicate that the rate of elimination of fluid by the contractile vacuole in *Amoeba proteus* is practically independent of the hydrogen-ion concentration except in the region of neutrality where it decreases markedly and that it gradually decreases to zero in hypertonic solutions.

Discussion

The results presented in Table I show that in amoebae which have been transferred from Ringer solution to sodium solution of various hydrogen-ion concentrations, there was a change in the rate of elimination of fluid by the contractile vacuole of only 0.07 per cent of the volume of the amoebae per minute. It is consequently obvious that the action of the contractile vacuole was only slightly involved in the changes in the volume of the amoebae observed, in relation to changes in hydrogen-ion concentration (Fig. 1), and that these changes were consequently largely due to the effect of the hydrogen-ion concentration of the solutions used, on the permeability of the surface layer to water.

Table I and other evidence presented above show that in the amoebae which had been transferred from 0.002 M Ringer solution to 0.002 M Ringer solution plus 0.2 M lactose, the rate of elimination of fluid by the contractile vacuole decreased from 0.3 per cent of the volume of the amoebae per minute to zero in an average of 18.5 minutes. No observations were made on the action of the vacuole in amoebae which had been transferred in the opposite direction, but it is highly probable that after such a transfer the vacuole becomes active as rapidly and to the same extent as it becomes inactive after the reverse transfer. If this is true, decrease in volume of amoebae in hypertonic solution is considerably augmented and increase in volume of amoebae in hypotonic solution is considerably retarded, owing to elimination of fluid by the vacuole; that is, the amount of fluid which leaves the amoebae directly through the surface in the hypertonic solution is equal to the increase in volume minus the amount eliminated by the vacuoles and the amount which enters the amoebae directly through the surface in the hypotonic solutions is equal to the increase in the volume of the amoebae plus the amount eliminated by the vacuoles.

Mast and Fowler (1935) calculated the permeability constant for water from results obtained in observations on the increase in the volume of amoebae in hypotonic solutions, but they did not consider the effect of the action of the contractile vacuole on the volume. As stated above, this probably amounted to 0.3 per cent of the volume of the amoebae per minute soon after the transfer to these solutions. The calculated value obtained by them (0.026) is therefore somewhat too small.

In the preceding paper it was demonstrated that after amoebae have been transferred from Ringer solution to this solution plus 0.2 M lactose, they decrease about 15 per cent in volume in 15 minutes and it was demonstrated above that under these conditions they continue to contract for about 18.5 minutes. It is therefore obvious that elimination of fluid through the vacuole continues after considerable fluid has passed out of the body by diffusion and that the action of the vacuole is not immediately dependent upon entrance of fluid and turgidity of the cell. This also obtains for other protozoa (Kitching, 1938, p. 148).

Pitts and Mast (1934), in observations on the gel/sol ratio in *Amoeba proteus*, obtained results which in general support the conclusion reached above, namely that the gel/sol ratio is relatively low in the region of neutrality. They also found that the rate of locomotion is low in this region and Table I above shows that the rate of elimination of fluid by the contractile vacuole is also low in this region. This indicates that the rate of locomotion and the action of the vacuole vary inversely with the fluidity of the cytoplasm and that it is maximum at neutrality. This is probably in some way correlated with the isoelectric point of a prominent protein in the cytoplasm.

The extraordinary changes observed in the gel/sol ratio in calcium solutions indicate remarkably rapid and extensive adjustment but concerning the processes in this adjustment there is no evidence.

Summary

1. As the hydrogen-ion concentration decreases from pH 5.5 the volume of *Amoeba proteus* in solutions containing sodium as the only metallic ion increases to a maximum at pH 6.0, then decreases to a minimum at pH 7.0, and then increases to a second maximum at pH 7.5; and the gel/sol ratio increases to a maximum at pH 6.5, then decreases very extensively to a minimum at pH 7.0, and then increases equally extensively to a second maximum at pH 7.5.

2. In solutions containing potassium as the only metallic ion the volume and the gel/sol ratio decrease to a minimum at pH 6.0 and then increase.

3. In solutions containing calcium as the only metallic ion the volume remains nearly constant, but the gel/sol ratio increases very rapidly and extensively and then gradually decreases; but the extent of change in this ratio varies directly with the hydrogen-ion concentration.

4. The rate of elimination of fluid by the contractile vacuole is practically independent of the hydrogen-ion concentration except in the region of neutrality where it decreases markedly. In hypertonic solutions it gradually decreases to zero.

5. The change in the rate of elimination of fluid by the vacuole in relation to hydrogen-ion concentration is so low in comparison with the change in rate of passage of fluid directly through the surface that it is negligible. The changes observed in the volume of the amoebae in relation to the hydrogen-ion concentration were therefore almost entirely due to changes in the rate of transfer of fluid directly through the surface, i.e. to changes in the permeability of the surface to water.

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